PLANT PHYSIOLOGY

EDITORS

ALLAN H. BROWN

MARY A. SWAEBLY-EHRLICH

R. S. BANDURSKI

S. B. HENDRICKS

T. C. BROYER

C. H. WADLEIGH

A. S. CRAFTS

K. V. THIMANN

J. MYERS

O. BIDDULPH

H. A. BORTHWICK

A. C. LEOPOLD

W. A. ANDREAE

P. J. KRAMER

R. H. BURRIS

D. R. GODDARD

S. A. GORDON

VOLUME 34

1959

Craftsmen, Inc., Kutztown, Pennsylvania

119351

TABLE OF CONTENTS

NUMBER 1. JANUARY

Stimulation of lettuce seed germination by 6-(substituted) purines. CHARLES G. SKINNER and WILLIAM SHIVE 1– 3

Influence of 2,4-dichlorophenoxyacetic acid on solute uptake by Chlorella. RANDOLPH T. WEDDING, LOUIS C. ERICKSON, and M. KAY BLACK 3– 10

Effects of atmospheric fluorides and various types of injury on the respiration of leaf tissue. A. C. HILL, M. R. PACK, L. G. TRANSTRUM, and W. S. WINTERS 11– 16

An analysis of auxin-gibberellin interaction in pea stem tissue. ARTHUR W. GALSTON and HAVA WARBURG 16– 22

Foliar uptake of salt constituents of water by citrus plants during intermittent sprinkling and immersion. FRANK M. EATON and R. B. HARDING 22– 26

Metabolic studies of applied indoleacetic acid-1-C^{14} in plant tissues as affected by light and 2,4-D treatment. S. C. FANG, PATRICIA THEISEN, and JOSEPH S. BUTTS 26– 32

The electron transfer system of skunk cabbage mitochondria. BRITTON CHANCE and DAVID P. HACKETT 33– 49

The response of the young tomato plant to a brief period of water shortage. IV. Effect of water stress on the ribonucleic acid metabolism of tomato leaves. C. T. GATES and JAMES BONNER 49– 55

Chloroplast isolation in nonaqueous media. C. RALPH STOCKING 56– 61

Effect of 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid on the geotropic and phototropic responses of seedlings of various species. LEO C. VANDER BEEK 61– 65

Effects of growth-regulating substances on the carbon dioxide evolution and post-harvest ripening of tomatoes. RICHARD T. HARTMAN 65– 72

Studies on sulfate utilization by *Chlorella pyrenoidosa* using sulfate-S^{35}; the occurrence of S-adenosyl methionine. JEROME A. SCHIFF 73– 80

Electron transport mechanisms in tobacco roots. I. Studies on the cytochrome and related systems. EDWARD C. SISLER and HAROLD J. EVANS 81– 90

News and notes 90– 93

Information and instructions for contributors to Plant Physiology 94– 96

NUMBER 2. MARCH

Effects of petroleum oils on the oxygen uptake in respiration of parsnip and mustard. CONSTANCE NOZZOLILLO and V. A. HELSON 97–100

Comparative responses of carbon dioxide outburst and uptake in tobacco. JOHN P. DECKER 100–102

Some effects of temperature and carbon dioxide concentration on photosynthesis of Mimulus. JOHN P. DECKER 103–106

Effect of acid scarification on lupine seed impermeability. ROBERT E. BURNS 107–108

Some factors which affect the synthesis of chlorogenic acid in disks of potato tuber. MILTON ZUCKER and CARL C. LEVY 108–112

Effect of 2,4-dichlorophenoxyacetic acid and 2,4-dinitrophenol on the uptake and metabolism of exogenous substrates by corn roots. T. E. HUMPHREYS and W. M. DUGGER, JR. 112–116

Effect of some substituted benzoic acids and related compounds on the distribution of callus growth in tobacco stem explants. GEORGE W. KEITT, JR. and FOLKE SKOOG. 117–122

Dark fixation and CO_2 by tobacco leaves. GEORGE KUNITAKE, CLYDE STITT, and PAUL SALTMAN 123–127

Internal distribution of coenzyme Q in higher plants. FREDERICK L. CRANE 128–131

Studies of the rest period. I. Growth, translocation, and respiratory changes in the embryonic organs of the after-ripening cherry seed.
BRUCE M. POLLOCK and HARVEY O. OLNEY 131–142
Formation of β-phenylglucoside in plant leaves.
C. W. NYSTROM, N. E. TOLBERT, and S. H. WENDER 142–143
Oxidation of indoleacetic acid by quackgrass rhizomes.
J. B. MUDD, B. G., JOHNSON, R. H. BURRIS, and K. P. BUCHHOLTZ 144–148
Malonate as a participant in organic acid metabolism in bush bean leaves.
R. H. YOUNG and L. M. SHANNON 149–152
Enzymatic degradation of adenosine triphosphate to adenine by cabbage leaf preparations. ..... MENDEL MAZELIS 153–158
Growth and development of isolated Phycomyces sporangiophores.
HANS E. GRUEN 158–168
Gibberellin in the induction of parthenocarpy in Zephyranthes.
R. C. SACHAR and MANJU KAPOOR 168–170
Metabolism and citrulline and ornithine in mung bean mitochondria. D. H. BONE 171–175
News and notes 176

NUMBER 3. MAY

Robert Emerson, obituary. ..... EUGENE RABINOWITCH 179–184
Light induced absorption changes in photosynthetic organisms. II. A split-beam difference spectrophotometer. ..... BESSEL KOK 184–192
Influence of selective light scattering on measurements of absorption spectra of Chlorella. ..... PAUL LATIMER 193–199
Chromatic transients in the photosynthesis of a green alga. ..... L. R. BLINKS 200–203
Induction of fluorescence in quinone poisoned Chlorella cells. .. JEAN LAVOREL 204–209
Quantum requirement for phosphopyridine nucleotide reduction in photosynthesis. ..... L. N. M. DUYSENS and J. AMESZ 210–213
Primary photochemical and photophysical processes in photosynthesis.
EUGENE RABINOWITCH 213–218
Further studies on the carbon dioxide burst in algae.
R. G. HILLER and C. P. WHITTINGHAM 219–222
Complexities of manometric measurement of changing rates of gas production.
G. E. BRIGGS 222–224
Relation between respiration and photosynthesis in the green alga, *Ankistrodesmus braunii*. ..... ALLAN H. BROWN and DALE WEIS 224–234
Kinetic relationships between photosynthesis and respiration in the algal flagellate, *Ochromonas malhamensis*. ..... DALE WEIS and ALLAN H. BROWN 235–239
Pyocyanine and phosphorylation with chloroplasts.
ROBIN HILL and D. A. WALKER 240–245
Photoreduction of chlorophyll a in the presence of ascorbic acid in pyridine solutions. ..... THOMAS T. BANNISTER 246–254
Photoreduction and photooxidation of cytochrome c by spinach chloroplast preparations. ..... R. H. NIEMAN and BIRGIT VENNESLAND 255–262
Fractionation and purification of cytochrome c photooxidase of spinach.
R. H. NIEMAN, H. NAKAMURA, and BIRGIT VENNESLAND 262–267
Comparison of three photochemical activities of chloroplasts.
JIMMY W. HINKSON and LEO P. VERNON 268–272
Uncouplers of spinach chloroplast photosynthetic phosphorylation.
DAVID W. KROGMANN, ANDRE T. JAGENDORF, and MORDHAY AVRON 272–277
Comparison of ferricyanide and 2,3',6-trichlorophenol indophenol as Hill reaction oxidants. ..... DAVID W. KROGMANN and ANDRE T. JAGENDORF 277–282
Stability of isolated chloroplast preparations and its effect on Hill reaction measurements. ..... THOMAS PUNNETT 283–289
A pyridine nucleotide-cytochrome c reductase isolated from chloroplasts.
KEN TAKAMATSU, MITSUO NISHIMURA, and HIROSHI TAMIYA 289–293
The mechanism of the photochemical activity of isolated chloroplasts. III. Dependence of velocity on light intensity.
JOHN S. RIESKE, RUFUS LUMRY, and JOHN D. SPIKES 293–300

The mechanism of the photochemical activity of isolated chloroplasts. V. Interpretation of the rate parameters. . . Rufus Lumry and John S. Rieske 301–305
Absorption spectra and relative photostability of the different forms of chlorophyll in Chlorella. ..... Jeanette S. Brown and C. Stacy French 305–309
Reduction of chlorophyllides, chlorophylls, and chlorophyll derivatives by sodium borohydride. ..... A. Stanley Holt 310–314
Chloroplast lipids as carbohydrate reservoirs.
A. A. Benson, J. F. G. M. Wintermans, and R. Wiser 315–317
Factors affecting light induced fixation of carbon dioxide by isolated spinach chloroplasts. ..... Martin Gibbs and Nona Calo 318–323
Intracellular and phylogenetic distribution of ribulose 1,5-diphosphate carboxylase and D-glyceraldehyde-3-phosphate dehydrogenases.
R. C. Fuller and Martin Gibbs 324–329
Biosynthesis and possible functional relationships among the carotenoids; and between chlorophyll a and chlorophyll b.
U. Blass, J. M. Anderson, and M. Calvin 329–333
Studies on nitrogen fixation and photosynthesis of *Rhodospirillum rubrum*.
Douglas C. Pratt and Albert W. Frenkel 333–337
Pigment carrying structures of the photosynthetic non-sulphur purple bacterium *Rhodopseudomonas spheroides*. ..... J. B. Thomas 338–340
Formation and bleaching of chlorophyll in albino corn seedlings.
James H. C. Smith, Lois J. Durham, and Charles F. Wurster 340–345
On the mass culture of algae. II. Yield as a function of cell concentration under continuous sunlight irradiance.
Jack Meyers and Jo-Ruth Graham 345–352
A sensitive stage in synchronized cultures of Chlorella.
A. Pirson, H. Lorenzen, and A. Koepper 353–355
Efficiency of light energy conversion in plant growth. ..... E. C. Wassink 356–361
News and notes 363

Number 4. July

Studies on auxin-membrane permeability relations in fruit and leaf tissues.
Joseph A. Sacher 365–372
Some metabolic responses of alfalfa seedlings to freezing.
C. R. Swanson and M. W. Adams 372–376
Metabolism of C^{14}-bicarbonate, P^{32}-phosphate, or S^{35}-sulfate by lettuce seed during germination. ..... Alan H. Haber and N. E. Tolbert 376–380
Mechanisms of action of polymyxin B on Chlorella and Scenedesmus.
R. A. Galloway and R. W. Krauss 380–389
On the occurrence of free-galacturonic acid in apples and tomatoes.
John H. McClendon, C. W. Woodmansee, and G. Fred Somers 389–391
Enzyme activation in wheat grains in relation to water content. Glutamic acid—alanine transaminase, and glutamic acid decarboxylase.
Pekka Linko and Max Milner 392–396
Methods for the extraction of enzymes from cereal leaves with especial reference to the triosephosphate dehydrogenases.
R. H. Hageman and E. R. Waygood 396–400
Effects of artificial increases in sugar content on frost hardiness. . . J. Levitt 401–402
Distribution and properties of isocitritase in plants.
W. D. Carpenter and Harry Beevers 403–409
Changes in endogenous growth substances during flower development.
H. Harada and J. P. Nitsch 409–415
Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. ..... D. C. McCune and A. W. Galston 416–418
Effect of light upon the behavior of citric acid in leaves of *Bryophyllum calycinum* Salisb. ..... Hubert Bradford Vickery 418–427
Respiratory and associated responses of carrot disc to substituted phenols.
B. K. Gaur and Harry Beevers 427–432

Studies on the mechanism of herbicidal action of 2-chloro-4,6-bis(ethylamino)-S-triazine.
D. E. Moreland, W. A. Gentner, J. L. Hilton, and K. L. Hill 432–435
Comparison of the nucleic acid, total nitrogen and protein nitrogen levels of normal and tumor tissue under similar growth conditions.
Hope H. Robson, Matthew, A. Budd, and Henry T. Yost, Jr. 435–440
Physiology of growth and reproduction in rice. II. Effect of plant growth substances on three winter varieties. ..... G. Misra and G. Sahu 441–445
Effects of iron and chelating agents on dark carboxylation reactions in plant homogenates.
R. C. Huffaker, D. O. Hall, L. M. Shannon, A. Wallace, and W. A. Rhoads 446–449
Loss of phosphorus-32 by plant roots after foliar application. Fred H. Emmert 449–454
Germination of light sensitive seed in crossed gradients of temperature and light.
Ruth F. Elliott and C. Stacy French 454–456
Studies on the mechanism of stem growth inhibition by visible radiation.
James A. Lockhart 457–460
Growth responses of Alaska pea seedlings to visible radiation and gibberellic acid. ..... James A. Lockhart and Virginia Gottschall 460–465
Endogenous substrates of dormant, activated, and germinating ascospores of *Neuraspora tetrasperma*. ..... B. T. Lingappa and A. S. Sussman 466–472
Influence of temperature on the response of germinating barley grains to potassium gibberellate. ..... Theodore W. Sudia 473–475
Phosphorus compounds of cotton embryos and their changes during germination.
David R. Ergle and Gene Guinn 476–481
Winter freezing in relation to the rise of sap in tall trees.
Bertiie Ruud Lybeck 482–486
News and notes 487

Number 5. September

Tryptophan synthetase activity in pea seedling extracts.
J. B. Greenberg and A. W. Galston 489–494
Identification of the anthocyanins in petals of tulip varieties Smiling Queen and Pride of Haarlem. ..... A. H. Halevy and S. Asen 494–499
Formation of embryo starch during germination as an indicator of viability and vigor in heat-damaged barley. ..... Richard C. French 500–505
Nitrogen and sulfur changes associated with growth uncoupled from cell division in *Chlorella vulgaris*. ..... Alex Shrift 505–512
Translocation of calcium in the bean plant.
O. Biddulph, R. Cory, and Susann Biddulph 512–519
Fixation of carbon dioxide in particulate preparations from barley roots.
J. S. D. Graham and L. C. T. Young 520–526
Carbon dioxide fixation and salt uptake in particulate preparations from barley roots. ..... L. C. T. Young and J. S. D. Graham 527–529
Effect of tissue age on hexose metabolism. I. An enzyme study with pea root.
Martin Gibbs and Joan M. Earl 529–532
An airflow planimeter for measuring the area of detached leaves.
H. V. Jenkins 532–536
Anthocyanin synthesis in corn endosperm tissue cultures. I. Identity of the pigments and general factors. ..... Jacob Straus 536–541
Amide metabolism in higher plants. III. Distribution of glutamyl transferase and glutamine synthetase activity. ..... W. D. Loomis 541–546
Isolation of two quinones with coenzyme Q activity from alfalfa.
Frederick L. Crane 546–551
Kinetics and properties of cytochrome c photooxidase of spinach.
N. I. Bishop, H. Nakamura, J. Blatt, and Birgit Vennesland 551–557
Activation of germination in Douglas fir seed by hydrogen peroxide.
Te May Ching 557–563

Alteration of the protein components and other constituents in the tobacco leaf by shading. ..... MILTON ZUCKER 563–569
Experimental modification of plant senescence.
A. C. LEOPOLD, ETHEL NIEDERGANG-KAMIEN, and JULES JANICK 570–573
Latitudinal effect on respiration in some northern plants.
SUSAN I. SCHOLANDER and JOAN T. KANWISHER 574–576
Factors in corn steep water promoting growth of plant tissues.
J. EUGENE FOX and CARLOS MILLER 577–579
Use of specifically labeled glucose and gluconate in the evaluation of catabolic pathways for glucose in corn roots.
T. E. HUMPHREYS and W. M. DUGGER, JR. 580–582
An inhibitor of oxalic acid oxidase in beet extracts.
B. J. D. MEEUSE and JEAN M. CAMPBELL 583–586
News and notes 586
Information and instructions for contributors to Plant Physiology 587–588

NUMBER 6. NOVEMBER

A soluble pyridine nucleotide oxidase system from barley roots.
JOHN A. ROMBERGER 589–597
Growth regulators and flowering. II. The cobaltous ion.
FRANK B. SALISBURY 598–604
Flowering in fruit trees and annual plants as affected by purines, pyrimidines and triiodobenzoic acid. ..... B. KESSLER, R. BAK, and A. COHEN 605–608
Molybdenum-manganese-iron antagonisms in the nutrition of tomato plants
GERALD C. GERLOFF, P. R. STOUT, and L. H. P. JONES 608–613
Further studies on comparative mobility of labeled herbicides. .. A. S. CRAFTS 613–620
Some observations on potassium accumulation in corn root mitochondria.
J. S. KAHN and J. B. HANSON 621–629
The aseptic culture of isolated buds of *Pseudotsuga taxifolia*.
KHALIL H. AL-TALIB and JOHN G. TORREY 630–637
An improved technique for the growth of Chlorella in $C^{14}O_2$.
PAUL D. ELLNER 638–640
The effects of triiodobenzoic acid and urea on the response of chlorotic lemon (*Citrus limonia*) trees to foliar application of iron compounds.
A. BAR-AKIVA and E. J. HEWITT 641–642
Effect of vernalization on heat resistance in two varieties of peas.
H. R. HIGHKIN 643–644
The possible role of adenosine triphosphate in rubidium absorption as revealed by the influence of external phosphate, dinitrophenol and arsenate.
N. HIGINBOTHAM 645–650
Ribulose 1,5-diphosphate carboxylase activity in relation to photosynthesis by intact leaves and isolated chloroplasts.
ROBERT M. SMILLIE and R. C. FULLER 651–656
Estimated contributions of root and shoot to the nicotine content of the tobacco plant. ..... R. F. DAWSON and MARIE L. SOLT 656–661
A wide-range A.C. bridge for determining injury and death.
C. V. DE PLATER and C. G. GREENHAM 661–667
Nitrogen mobilization in pea seedlings.
JOHN M. LAWRENCE, KATHERINE M. DAY, and JANET E. STEPHENSON 668–674
Bound water and frost hardiness. ..... J. LEVITT 674–677
News and notes 678
General Index, Volume 34 679–692

SUPPLEMENT. i–xxii

Corrections

Page 259, legend for fig 3, third line. The curves should be identified as follows: "Curve 1: 0.3 μM of KCN; curve 2: no KCN."

Page 108, line 8 from bottom and line 11 from bottom. The name Kuc should be spelled Kuc.

Page 112, literature references 5 and 6. The name Kuc should be spelled Kuc.

Page 8, table III. The subheadings of this table should read as follows:

2,4-D Concentration, molar	Increase in absorbance during 16-hour incubation after treatment	Increase in absorbance during additional 32-hour incubation

Page 8, second col. line 9. The line should read, "the external solution: 1) Mannitol at 25° C, sodium."

PLANT PHYSIOLOGY

VOLUME 34 JANUARY, 1959 NUMBER 1

STIMULATION OF LETTUCE SEED GERMINATION BY 6-(SUBSTITUTED) PURINES[1]

CHARLES G. SKINNER AND WILLIAM SHIVE

CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AUSTIN, TEXAS

Presoaking several varieties of seed, especially lettuce, in various 6-(substituted)amino- and thiopurine solutions has been found to increase their rate of germination (3, 5, 6). Recently it was also reported that these purine derivatives are synergistic with gibberellin in inducing these biological responses whereas gibberellin alone was only moderately active in stimulating germination of Early Curled Simpson lettuce seed (7, 8). The mode of biochemical action of these purine derivatives and gibberellin (2) in affecting the rate of germination is as yet unknown, although it has been observed that seed activation by white light is augmented by pretreatment of the seed with various 6-(substituted)purine solutions (6). Recently Miller (4) demonstrated that maximal purine-activation of Grand Rapids lettuce seed germination is somewhat dependent on the presence of light. The action spectrum for the influence of light on germination is not necessarily the same in the presence of 6-(substituted) purines as in their absence (1); far-red light initiates appreciable germination after pretreatment with kinetin, even though the far-red light is less effective than red light.

In the present paper, a study was made of the minimal contact time required for effective activation as initiated by presoaking lettuce seed in 6-benzylaminopurine solutions, or in a synergistic mixture of benzylaminopurine and gibberellin. The effect of the presence or absence of light on the rate of germination of pretreated lettuce seed also was determined.

MATERIALS AND METHODS

Lettuce seeds (Early Curled Simpson) were placed in the appropriate solution of 6-benzylaminopurine, gibberellin, or a combination of both in the presence of a dim blue safety light (fluorescent light filtered through 3 layers of du Pont MSC Dk. Blue cellophane), and quickly shaken to wet the seed. The seeds were then placed in the dark for the additional time period of soaking noted for each treatment. Seeds were removed from each solution tested after soaking for 1, 3, 10, 30 and 60 minutes, immediately washed 3 times with distilled water to remove any excess purine solution, and carefully blotted dry between pieces of filter paper. The seeds were then placed in Petri dishes containing filter paper wet with water, and allowed to germinate at 30° C in the dark for 24 or 48 hours. Approximately 100 seeds were used for each assay.

The above procedure was repeated except that the source of light under which the mechanical operations were carried out was varied according to the conditions being studied, and in all of these experiments the seeds were presoaked for 1 hour. The light conditions studied included (a) "normal" conditions as above, (b) "reflected" blue light in which an opaque board was placed directly under the blue light source so that only a minimal amount of reflected blue light reached the seeds during the mechanical operations, (c) same as (b) except that after the seeds were placed in the Petri dishes they were exposed to 1 minute of far-red light from a 50-watt tungsten bulb filtered through 3 layers of du Pont 300 MSC red cellophane alternated with 2 layers of 300 MSC blue cellophane (3) prior to germination in the dark at 30° C, and (d) all of the mechanical operations were carried out in darkness so that the wet seeds were protected from any light exposure prior to the final reading after the germination period.

RESULTS AND DISCUSSION

It has previously been reported that it makes no appreciable difference in the ultimate percentage of lettuce seed germination whether the seeds are pretreated with the purine solution during the 1st or final hour of an 8 hour total presoaking treatment (8). It seemed of interest to determine the minimum length of time required for presoaking the seeds in these purine solutions to initiate germination activity. The results presented in figure 1 indicate that the activation is initiated in a relatively short period of time (1 min). Since the seeds were washed 3 times with water and blotted dry following the various treatment intervals, it seems unlikely that any appreciable amount of purine derivative remained in the water adhering to the outside of the seed coat. Lettuce seed does not imbibe sufficient water in these short intervals to promote maximal germination. For example, with

[1] Received June 2, 1958.

TABLE I

LASTING EFFECT OF PRETREATMENT OF LETTUCE SEEDS WITH 6-(SUBSTITUTED)PURINE SOLUTIONS ON RATE OF GERMINATION*

PRETREATMENT CONDITIONS	% OF SEEDS GERMINATED AFTER DRY STORAGE FOR:**	
	5 days	25 days
None	0	0
Water	2	2
6-Benzylaminopurine, 3 μg/ml	93	93
6-Benzylaminopurine, 3 μg/ml + gibberellin, 100 μg/ml	89	94

* Seeds presoaked in indicated solution for 1 hour, dried in vacuo for noted time period, and subsequently placed on filter paper wet with water for germination at 30° C in the dark.

** Percent germination values determined after 48 hours, 100 or more seeds used per assay.

seeds presoaked for 1 hour in water, about 32 % had germinated after 24 hours at 30° C even when exposed to white light. With seeds presoaked for only 10 minutes in a solution of 6-benzylaminopurine (3 μg/ml), 36 % had germinated at 30° C in the dark. With seeds pretreated in a solution containing both 6-benzylaminopurine (3 μg/ml) and gibberellin (100 μg/ml), germination reached 56 %. Gibberellin alone had no appreciable effect on germination under the conditions of this test.

The pretreated lettuce seeds will remain in an activated state for a relatively long period of time. To demonstrate this effect, 4 groups of seeds were subjected to the pretreatment conditions indicated in table I, and then dried and kept in a vacuum desiccator for the time periods noted in the table. The seeds pretreated with either 6-benzylaminopurine or the synergistic combination of 6-benzylaminopurine and gibberellin, retained their germination activity when subsequently placed on filter paper wet with water for germination in the dark at 30° C. Treated seeds kept for about 1 month and then brought into contact with water exhibited essentially complete germination within 48 hours. Control seeds pretreated with water only, did not show enhanced germination under the same conditions.

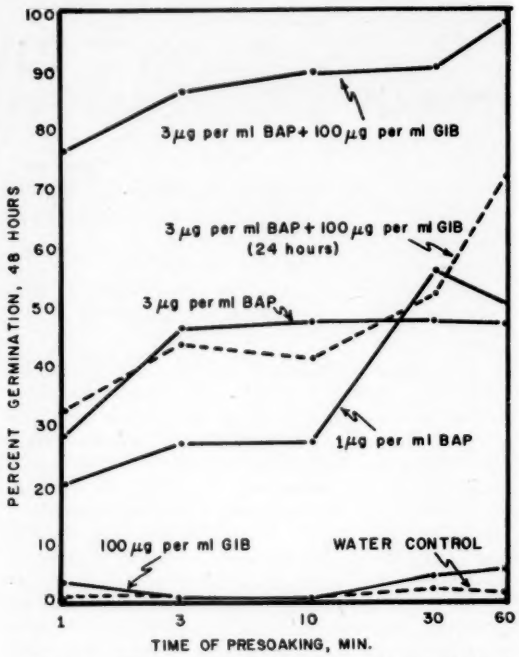


FIG. 1. Effect of presoaking time on germination of lettuce seed. Early Curled Simpson lettuce seeds were pretreated with the various solutions (BAP = 6-benzylaminopurine, GIB = gibberellin) for the indicated time period, carefully washed, and allowed to germinate in Petri dishes on filter paper wet with water at 30° C in the dark; 100 or more seeds were used in each assay.

Light has previously been reported to augment the effect of 6-(substituted)purines on increasing the rate of seed germination (4, 6). About 50 % of the Early Curled Simpson lettuce seeds soaked in a physiologically active concentration of 6-benzylaminopurine

TABLE II

EFFECT OF VARIOUS LIGHT SOURCES ON GERMINATION OF PURINE-TREATED EARLY CURLED SIMPSON LETTUCE SEEDS*

PRETREATMENT SOLUTION	LIGHT TREATMENT:	% OF SEEDS GERMINATED IN 24 HR**				% OF SEEDS GERMINATED IN 48 HR**			
		D[a]	FRL[b]	B[c]	RB[d]	D[a]	FRL[b]	B[c]	RB[d]
Water blank control		3	0	0	0	1	3	3	0
3 γ/ml 6-benzylaminopurine		45	99	41	46	76	97	62	75
10 γ/ml 6-benzylaminopurine		13	72	23	18	57	94	57	60
30 γ/ml gibberellin		7	7	2	0	10	17	7	9
100 γ/ml gibberellin		12	9	8	4	19	27	8	14
3 γ/ml 6-benzylaminopurine + 100 γ/ml gibberellin		76	95	73	72	94	93	95	95

* These data are averages of at least 2 assays containing 100 or more seeds each.

** Light conditions: [a] dark conditions; [b] far-red light; [c] blue light; [d] reflected blue light.

germinated within 24 hours after prior exposure either to blue light or when maintained under complete darkness. However, far-red light augmented the germination of seeds pretreated with 6-benzylaminopurine so that nearly all such seeds germinated within 24 hours after treatment (table II). Far-red light also augmented the rate of germination of seed pretreated with a synergistic mixture of 6-benzylaminopurine and gibberellin. Lowering the temperature of germination to 25° C caused an appreciable increase in the rate of germination and augmented the synergistic effect of purines and gibberellin in combination. Such alternate stimuli for augmenting the same biological response could serve as protective mechanisms to insure germination of a certain percentage of seeds even under unfavorable environmental conditions.

Summary

Presoaking Early Curled Simpson lettuce seeds in physiologically active concentrations of 6-benzylaminopurine or a synergistic mixture of the purine derivative and gibberellin for only 1 minute will significantly enhance germination, when the seeds are subsequently washed and placed in a dark germinator or at 30° C for 48 hours. This activation of germination is comparable to that observed by exposure of wetted lettuce seeds to red-light. The activation of purine-treated Early Curled Simpson lettuce seeds is augmented by far-red light; but, in contrast, there is no appreciable difference in the rate of germination of these seeds between treatments involving exposure to blue light or complete dark conditions during pregermination handling. The far-red light also augments the germination of seed pretreated in a synergistic combination of 6-benzylaminopurine and gibberellin.

Literature Cited

1. Borthwick, H. A., Hendricks, S. B., Parker, M. W., Toole, E. H. and Toole, Vivian K. A reversible photoreaction controlling seed germination. Proc. Natl. Acad. Sci., U.S. 38: 662–666. 1952.
2. Kahn, A. and Gross, J. A. Effect of gibberellin on germination of lettuce seed. Science 125: 645–646. 1957.
3. Miller, C. O. Similarity of some kinetin and red light effects. Plant Physiol. 31: 318–319. 1956.
4. Miller, C. O. The relationship of the kinetin and red light promotions of lettuce seed germination. Plant Physiol. 33: 115–117. 1958.
5. Skinner, C. G., Claybrook, J. R., Talbert, F. D. and Shive, W. Stimulation of seed germination by 6-(substituted) thiopurines. Arch. Biochem. Biophys. 65: 567–569. 1956.
6. Skinner, C. G., Claybrook, J. R., Talbert, F. and Shive, W. Effect of 6-(substituted) thio- and amino-purines on germination of lettuce seed. Plant Physiol. 32: 117–120. 1957.
7. Skinner, C. G. and Shive, W. Synergistic effect of gibberellin and 6-(substituted) purines on germination of lettuce seed. Arch. Biochem. Biophys. 74: 283–285. 1958.
8. Skinner, C. G., Talbert, F. D. and Shive, W. Effect of 6-(substituted) purines and gibberellin on the rate of seed germination. Plant Physiol. 33: 190–194. 1958.

INFLUENCE OF 2,4-DICHLOROPHENOXYACETIC ACID ON SOLUTE UPTAKE BY CHLORELLA[1, 2]

RANDOLPH T. WEDDING, LOUIS C. ERICKSON AND M. KAY BLACK

Department of Plant Biochemistry, University of California, Citrus Experiment Station, Riverside, California

Auxins, particularly indoleacetic acid, are well known for their ability to influence the distribution of water and solutes within individual cells and in tissues or intact plants. Some of these effects have been attributed to changes in cell permeability resulting from auxin application (1, 2, 5, 6, 7, 8, 10), and direct measurements of changes in uptake of water and solutes under the influence of externally applied auxins have been made (1, 7, 11, 13). Most studies of this subject, however, have consisted of measurements of plasmolysis-deplasmolysis time in hyper- and hypo-tonic solutions of various compounds, some of which were assumed to penetrate the cells readily, while others were assumed to be incapable of penetrating the cell under the conditions of the experiments. However, none of the literature available to us presents evidence for auxin effects on permeability based on actual measurement of solute uptake by cells under conditions which minimize or preclude secondary influences of the applied auxin on the measured quantity through changes in the metabolic activity of the cell. Since auxins are known to have profound effects on plant metabolism, experiments designed to measure changes in permeability due to auxins should eliminate as nearly as possible any influence of metabolism on the measurements made.

In these studies we have attempted to attain this objective by investigating the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the uptake of radioactive-tracer-labeled compounds under conditions which provide for minimum metabolic use or accumulation of the tracer within the cell.

[1] Received for publication June 12, 1958.
[2] Paper no. 1049, University of California Citrus Experiment Station, Riverside, California.

Materials and Methods

Chlorella pyrenoidosa Chick. (Emerson's strain) was grown as described previously (4), except that iron was provided in a chelated form rather than as ferric tartrate. Cell suspensions for uptake studies were routinely prepared by adjusting the concentration of cells in an aliquot of nutrient solution withdrawn from the large culture tube. The cell suspensions were diluted or centrifuged as required to give an absorbance value of 0.85 at 525 mμ. This standard suspension was concentrated 16 times by centrifuging successive aliquots into the same tube. The cells were then rinsed by resuspending in redistilled water and centrifuging. Final cell preparation was obtained by suspension in an appropriate volume of water; 1-ml aliquots were then distributed to 10-ml round-bottom tubes which were again centrifuged and the supernatant liquid discarded.

Treatments were made by adding 1 ml of water or 2,4-D solution, quickly suspending the packed cells by shaking with a "tube buzzer" and adding 1 ml of 0.02 M potassium phosphate buffer at pH 5.5 containing the desired radioactive compounds. The cells were again suspended with the buzzer and placed in a shaking water bath maintained at the desired temperature. In uptake experiments carried out at 25° C the cells were illuminated at a level of 80 to 90 ft-c by fluorescent lamps. Where uptake was studied at 1° C the cells were in complete darkness during the treatment and uptake periods.

Zero time for a given tube was taken as the time at which the buffer containing the labeled compound was added to the cell suspension. At 2.5 minutes prior to the end of the desired time interval the tubes were removed from the water bath, the cells were centrifuged down, and the supernatant liquid was decanted. The cells were rinsed quickly with redistilled water, centrifuged, and killed by adding 1 ml of butylamine or propylamine. The method was otherwise the same as described previously (15) except for variations in the counting method appropriate to the isotope used.

Two to 6 replicates of each treatment were run simultaneously, and additional replication was obtained by repetition of the entire series of treatments. Figures presented here are means of at least 4 and, in most cases, of 10 or more replicates of the same treatment.

The total volume of cells was determined by centrifuging aliquots of the suspension into capillary tubes at a centrifugal force of 150 G. In all cases the external concentrations reported are those in the final 2 ml of liquid containing the cells, but it is obvious that the actual concentrations are lower by the volume of rinse water which adhered to the cell pack and could not be removed. This volume is estimated to be less than 0.1 ml, and the dilution effect could not be demonstrated by assay of radioactive solutions added to the cells.

Experiments with Uniformly Labeled Sucrose-C^{14}: For studies of sucrose uptake, a uniformly labeled compound was prepared by purification from Canna photosynthate. The 80 % ethanol extract of the Canna leaf was separated chromatographically, and subsequent paper chromatography of the eluted sucrose indicated that no radioactive contaminants were present. The purified sucrose-C^{14} was dissolved in redistilled water and for these experiments was made to a concentration of 6.5 micromoles/liter in the presence of the Chlorella cells.

Preliminary experiments with sucrose indicated that at 25° C a very large fraction of the total sucrose entering the cells was metabolized even within short time intervals; all further sucrose uptake studies therefore were conducted at a temperature of 1° C. For these experiments all equipment and operations were transferred to a cold room maintained at that temperature, and the live cells did not rise above this temperature at any time after the concentrated cell suspension was prepared. Even at a temperature of 1° C, a portion of the sucrose was metabolized by Chlorella. Measurements of the magnitude of this activity were made with the use of Thunberg tubes containing 1 ml of 20 % KOH in the side arm. The Chlorella suspensions were incubated in these tubes for periods as long as 4 hours, and the $C^{14}O_2$ was determined by precipitating $BaC^{14}O_3$ from the KOH solution at the end of the experiment, filtering out the insoluble precipitate and counting the radiation from C^{14}. Determinations made in this way are expressed as micromoles of sucrose respired.

Experiments with Uniformly Labeled Mannitol-C^{14}: The labeled mannitol was prepared by the sodium borohydride reduction of fructose-C^{14} purified chromatographically from Canna photosynthate. The reduction products were separated by chromatography and the band enriched in mannitol but containing an impurity of sorbitol was dissolved in redistilled water and used in these studies. The final concentration of mannitol in the presence of Chlorella cells was 6.5 micromoles/liter. The mannitol uptake studies were carried out at 25° C, partly because of the relatively slow uptake of this material and partly because respiration measurements made in darkness with mannitol showed that less than 1 % of the total within the cells appeared as $C^{14}O_2$ during exposures of up to 2 hours at this temperature.

Experiments with $P^{32}O_4$: Phosphate uptake was studied simultaneously with that of sucrose. The phosphate buffer containing sucrose-C^{14} for the sucrose uptake experiments described above was fortified by the addition of 10^{-3} M $P^{32}O_4$. After drying on planchets, the cells and cell contents were assayed for total radioactivity. The stainless-steel planchets containing the cell preparations were ashed for 2 hours at 550° C and then recounted. The activity remaining after ashing was due to P^{32}, and the original total minus this amount represented C^{14} activity from the sucrose. The total phosphate concentration in the presence of the cells was 0.01 M.

Experiments with $S^{35}O_4$: For study of sulfate uptake, $Na_2S^{35}O_4$ at a concentration of 7.07 micromoles/liter was added to the potassium phosphate buffer. Since a very large metabolic uptake of sulfate was found at 25° C, all further experiments with this material were conducted at 1° C.

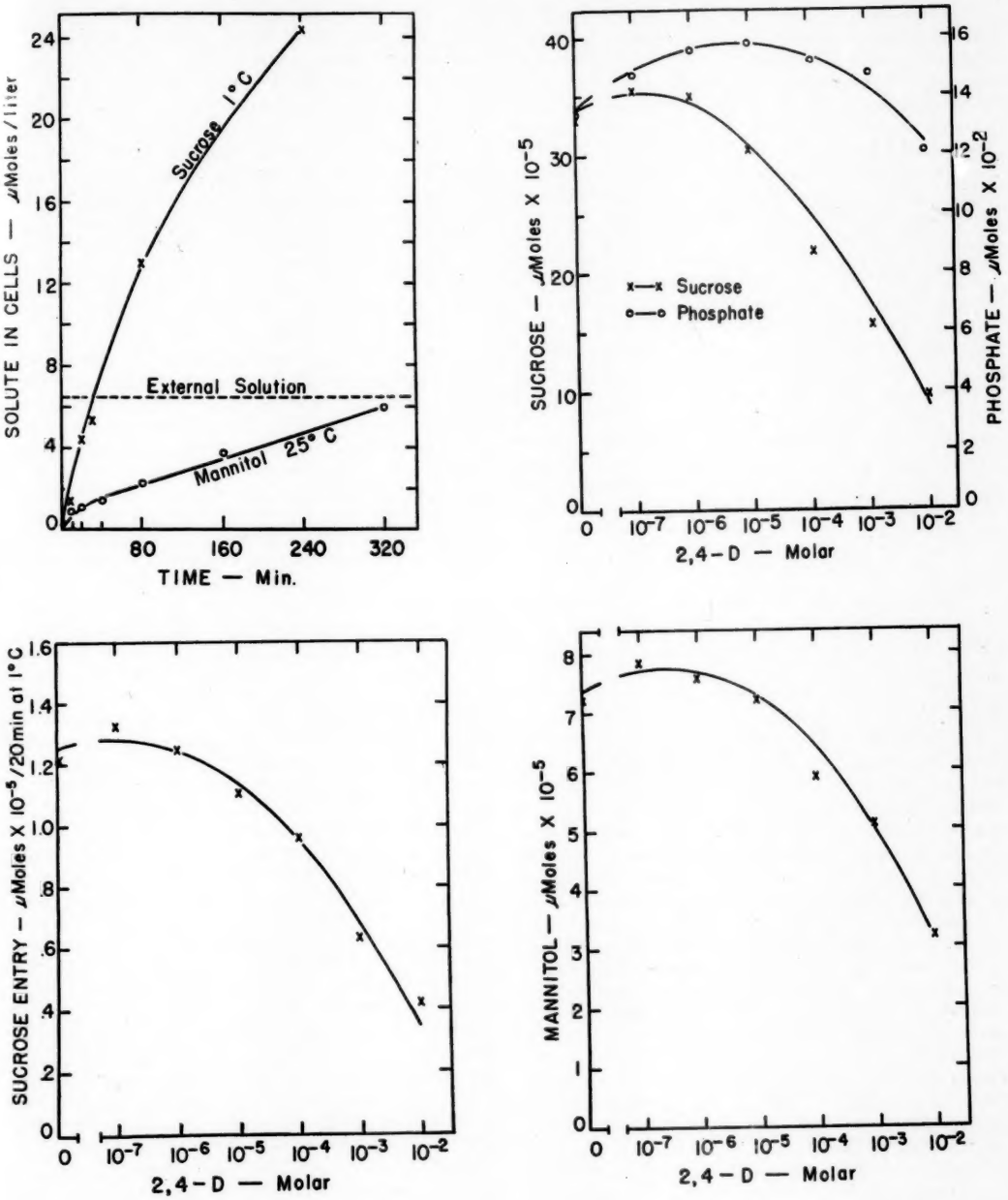


FIG. 1 (*upper left*). Time-course of the uptake of labeled sucrose and mannitol. Concentration in cells calculated on basis of total volume of cells packed at 150 G.

FIG. 2 (*lower left*). Effect of 2,4-D on permeability of chlorella to sucrose, measured by difference in rates of $C^{14}O_2$ production when sucrose-C^{14} is taken up prior to 2,4-D treatment and simultaneously with exposure to 2,4-D.

FIG. 3 (*upper right*). Effect of 2,4-D on uptake of sucrose and phosphate by Chlorella at 1° C. Uptake for 20-minute time interval from a solution containing 6.5 micromoles/liter of sucrose-C^{14} and 0.01 M $P^{32}O_4$.

FIG. 4 (*lower right*). Effect of 2,4-D on uptake of mannitol by Chlorella at 25°C. Uptake for 20-minute time interval from a solution containing 6.5 micromoles/liter of mannitol-C^{14}.

EXPERIMENTS WITH Na^{22}: For investigation of sodium uptake, $Na^{22}Cl$ at a concentration of 74.5 micromoles/liter in phosphate buffer was used. Studies with this ion were carried out at both 25° C and 1° C.

CHLORELLA SURVIVAL STUDIES: In order to determine whether the 2,4-D caused the death of any significant number of cells, the growth response of Chlorella to 2,4-D treatments like those used in uptake studies was investigated. In these experiments cells were prepared in the usual way and treated with 2,4-D in 0.01 M potassium phosphate buffer at pH 5.5 for periods of 20 minutes to 4 hours. At the end of the exposure period, the cells were centrifuged, rinsed twice with redistilled water, and resuspended in nutrient solution. The absorbance of the suspensions was determined at 525 mμ immediately after treatment and at intervals for 4 days. The increment of increased density found for each treatment is considered an approximate measure of the number of viable cells present at the termination of the treatment. Earlier experiments in our laboratory have shown that the rate of increase in the absorbance of chlorella suspensions is proportional to innoculum size for at least several days.

RESULTS

TIME-COURSE OF SUCROSE AND MANNITOL UPTAKE: The uptake of sucrose-C^{14} and mannitol-C^{14} as a function of time was investigated under the same conditions as those of the 2,4-D studies discussed below. The results of these experiments are shown in figure 1. It may be seen that sucrose penetrates Chlorella cells readily, reaching a concentration inside the cell equal to the external solution within 40 to 50 minutes in these studies. The further increase in internal sucrose concentration indicates that even at a temperature of 1° C an appreciable conversion of sucrose to nondiffusible forms takes place.

Mannitol, on the other hand, penetrates Chlorella cells slowly, even at 25° C. Only after 320 minutes, the longest time period used, could the mannitol be said to have come to approximate equilibrium with the external solution. The effect of metabolism on mannitol uptake is extremely small, as indicated by both respiration measurements and the slow rate of mannitol appearance within the cells. This slow rate of penetration and low order of metabolic use at normal room temperatures makes labeled mannitol a superior material for study of permeability changes divorced from indirect effects through changes in metabolism.

EFFECTS OF 2,4-D ON SUCROSE-C^{14} UPTAKE BY CHLORELLA: Since efforts to eliminate metabolic use of sucrose by Chlorella through the addition of sublethal amounts of KCN and NaF and by use of low temperatures had not been completely successful, an indirect measurement of the influence of 2,4-D on permeability to sucrose was attempted. This was done by determining the effect of 2,4-D on the production of $C^{14}O_2$ by Chlorella from sucrose-C^{14} taken up before 2,4-D treatment, and comparing this with $C^{14}O_2$ production from sucrose-C^{14} taken up simultaneously with the auxin treatment. To do this, Chlorella cells were first allowed to take up sucrose-C^{14} at 1° C for 1 hour, and then were centrifuged, rinsed, resuspended in buffer with various concentrations of 2,4-D, and transferred to Thunberg tubes. One ml of KOH in the side arm provided an absorbing medium for the $C^{14}O_2$. The Thunberg tubes were then shaken at 1° C for periods of 20 minutes to 4 hours, and the $C^{14}O_2$ produced during this time was determined. Under these conditions, the effect of 2,4-D would be primarily on metabolic production of CO_2, the only permeability factor involved being a possible change in outward movement of sucrose. Preliminary experiments indicated that the effect of 2,4-D on loss of sucrose by Chlorella was much less than that on uptake, so that this factor should be of relatively small magnitude.

To provide a comparison with these results, additional groups of cells were incubated at 1° C in Thunberg tubes with identical concentrations of 2,4-D, but with the sucrose-C^{14} in the solution at the same time as the 2,4-D. Under these conditions 2,4-D affected both the rate of penetration of the sucrose and the production of $C^{14}O_2$ by the cells. When the rate of respiration determined this way for a given concentration of 2,4-D is subtracted from the rate in the presence of the same concentration of 2,4-D but with sucrose taken up before the 2,4-D addition, the difference is an indication of the relative effect of that concentration of 2,4-D on the rate of sucrose entry into the cell. Because of the different lengths of time during which the cells were utilizing sucrose-C^{14} under the different conditions, rates of CO_2 production were first converted to percentages of the rate for the untreated cells in each condition, the percentages used for subtraction, and the residual percentage converted back to actual amount of sucrose in terms of the untreated cells of the group with sucrose and 2,4-D present simultaneously. These differences are presented in graphical form in figure 2. The respiration rates for the cells to which 2,4-D was applied after sucrose uptake are presented in table I. The inhibition of sucrose respiration shown here is somewhat at variance with the stimulation of endogenous Chlorella respiration by 2,4-D shown previously (16). However, comparison of these data with the previous work shows that the change in respiratory response with an increase in 2,4-D concentration is similar, with the rate tending to approach a maximum value at 10^{-4} to 10^{-3} M 2,4-D. The difference lies in the fact that in the present work this maximum does not exceed the rate for the untreated cells, while the earlier data showed an actual stimulation by 2,4-D. The previous data were obtained at pH 4.5 and 25° C which together with substrate differences may account for the lack of stimulation found here.

Examination of figure 2 indicates that the influence of 2,4-D on sucrose uptake measured in this indirect way consists of a small increase at low concentrations of 2,4-D with a progressive change in the direction of less uptake at higher concentrations. The data are presented in terms of $C^{14}O_2$ production for a 20-minute period to facilitate comparison with other data, but the determinations were actually made with a 4-hour incubation period. This was necessary Be-

cause the shorter time intervals did not provide sufficient $C^{14}O_2$ to permit accurate assay of radioactivity. The use of such interpolated data seems justified by the fact that measurements of $C^{14}O_2$ production by Chlorella made under these conditions indicate that this production is linear with time.

A more direct measurement of 2,4-D effects on the uptake of sucrose-C^{14} by Chlorella was also made, using the amount of sucrose entering the cells as an index of changes in permeability. These experiments were also conducted at 1° C. The rate of $C^{14}O_2$ production at various concentrations of 2,4-D determined above were used to make a partial correction for metabolic use of sucrose in the uptake experiments. For this purpose respiratory CO_2 production from sucrose was subtracted from the total sucrose uptake by Chlorella at each 2,4-D concentration. This corrects only for a fraction of the metabolized sucrose; the application of larger respiratory corrections would tend to accentuate the maximum and minimum figures for total sucrose uptake, since the trend of 2,4-D effects on respiration is generally the opposite of effects on sucrose uptake except at the highest concentrations (see table I and fig 2).

TABLE I

$C^{14}O_2$ PRODUCTION FROM SUCROSE-C^{14} AT 1° C WHEN CELLS ARE FIRST ALLOWED TO TAKE UP SUCROSE-C^{14} AND THEN TREATED WITH 2,4-D

2,4-D CONCENTRATION, MOLAR	SUCROSE-C^{14} RESPIRED IN 20 MIN ($\mu M \times 10^{-5}$)
0	2.05
10^{-7}	1.41
10^{-6}	1.68
10^{-5}	1.74
10^{-4}	1.91
10^{-3}	1.93
10^{-2}	1.20

The amount of sucrose-C^{14} found within Chlorella cells after 20-minute uptake while exposed to various concentrations of 2,4-D at 1° C is shown in the lower curve of figure 3. It will be noted that the general shape of the uptake curve is quite similar to that given by the indirect measurement used to obtain the data of figure 2. Each of these curves is best fitted by a second-order parabola when the amount of sucrose inside the cells is plotted against the $\log_{10}$ of 2,4-D concentration in the external medium.

EFFECTS OF 2,4-D ON MANNITOL-C^{14} UPTAKE BY CHLORELLA: When Chlorella suspensions were incubated in the dark with mannitol-C^{14} at 25° C for periods as long as 2 hours, less than 1 % of the total radioactivity found inside the cells could be recovered as $C^{14}O_2$. Because of the method of synthesis used for the mannitol, this amount of respired material might have come from an impurity of fructose in the preparation, but in any case it represents an extremely low rate of metabolism of the absorbed solute. Since metabolic use was at such a low level, mannitol uptake should reflect with some accuracy the alterations in permeability brought about by 2,4-D. The results of mannitol uptake studies are presented in figure 4, where it may be seen that permeability to mannitol appears to be affected by 2,4-D in much the same fashion as permeability to sucrose, with a small increase in uptake at low 2,4-D cencentrations followed by a progressive decrease in the amount of mannitol penetrating into the Chlorella cells at higher 2,4-D concentrations. This relationship is also best expressed by a second-order parabola.

EFFECTS OF 2,4-D ON $P^{32}O_4$ UPTAKE BY CHLORELLA: The uptake of $P^{32}O_4$ was studied simultaneously with that of sucrose in experiments mentioned earlier. Since the buffer used contained 0.01 M phosphate, the total concentration was much higher than that of the sucrose, and the amounts of phosphate found in the cells were correspondingly greater, as shown in the upper curve of figure 3. In the 20-minute uptake periods used, the phosphate within the cells had come to only 27 % of the external concentration. Therefore it is considered that the phosphate uptake shown in figure 3 is primarily influenced by changes in permeability of Chlorella due to 2,4-D.

The relation between 2,4-D and phosphate uptake is markedly different from that found between 2,4-D and sucrose or mannitol uptake. The amount of phosphate entering the cells is increased more by low concentrations of 2,4-D, and the increased uptake extends over a broader range of concentration, with 10^{-2} M, 2,4-D resulting in only a slight inhibition of uptake. The curve is best expressed by a second-order parabola, but the equation for the line is significantly different from that found for sucrose and mannitol (see Discussion and Conclusions).

EFFECTS OF 2,4-D ON $S^{35}O_4$ AND Na^{22} UPTAKE BY CHLORELLA: The effects of 2,4-D on the uptake of sulfate and sodium by Chlorella at 1° C are summarized in table II. It may be seen that under these conditions, there is no influence of 2,4-D on the uptake of either of these ions; this is further indicated by the fact that the line of best fit for each of these sets of data is a straight line with a slope which does not differ significantly from zero.

TABLE II

EFFECTS OF 2,4-D ON UPTAKE OF $S^{35}O_4$ AND Na^{22} BY CHLORELLA*

2,4-D CONCENTRATION, MOLAR	S^{35} IN CHLORELLA CELLS AFTER 20-MIN ($\mu M \times 10^{-5}$)	Na^{22} IN CHLORELLA CELLS AFTER 20-MIN ($\mu M \times 10^{-4}$)
0	2.62	9.12
10^{-7}	2.40	9.12
10^{-6}	2.28	8.72
10^{-5}	2.27	9.08
10^{-4}	2.52	9.41
10^{-3}	2.34	9.52
10^{-2}	2.80	9.36

* Uptake at 1° C. From solutions containing 7.07 micromoles/liter $S^{35}O_4$ and 74.5 micromoles/liter Na^{22}.

SURVIVAL OF 2,4-D-TREATED CHLORELLA: In spite of the pronounced differences found in the uptake of various organic and inorganic solutes under the influence of 2,4-D, there remained a possibility that all or part of the inhibition of uptake caused by high 2,4-D concentrations might have been due to killing of some fraction of the cells in the treated suspension. If this were the case, the decrease in the amount of material found within the cell could be attributed, not to a decrease in the rate of solute passage across the membrane due to a change in the resistance of the membrane to solute movement into the cell, but rather to a complete loss of semipermeability. The cells would then come to equilibrium with the external solution, and the rinse after treatment would remove most of the solute, thus causing an apparent decrease in uptake.

To check this possibility several experiments were performed in which cell suspensions were treated in the same way as for the uptake studies. At the end of various time periods the cells were centrifuged out of the 2,4-D solutions, rinsed twice with redistilled water, and suspended in nutrient solution. These treated cell suspensions were incubated under artificial illumination of 1000 ft-c for several days, and the optical absorbance of the suspensions was determined at intervals. If significant numbers of cells had been killed by the treatment, the rate of subsequent growth would have been reduced. The results of one of these experiments are summarized in table III. In this case the cells

TABLE III

INCREMENT IN ABSORBANCE AT 525 M_μ OF CHLORELLA CELL SUSPENSIONS FOLLOWING THEIR EXPOSURE TO 2,4-D AT 1° C

2,4-D CONCENTRATION, MOLAR	INCREASE IN ABSORBANCE DURING AFTER TREATMENT	INCREASE IN ABSORBANCE DURING INCUBATION
0	.288	.158
10^{-7}	.211	.159
10^{-6}	.233	.111
10^{-5}	.200	.163
10^{-4}	.223	.147
10^{-3}	.198	.175
10^{-2}	.212	.157

were exposed to 2,4-D for 4 hours at 1° C, and the figures presented are the increments in absorbance attained by the cells of each treatment after 16 and 48 hours incubation under conditions favorable for growth. These figures show little apparent effect of higher concentrations of 2,4-D in reducing Chlorella growth subsequent to treatment even after a 4-hour exposure and indicate that the decrease in uptake resulting from 2,4-D treatments probably is not due to death of the cells.

COMPARATIVE RATES OF SOLUTE UPTAKE BY CHLORELLA: Some information collected in the course of this work does not bear directly on the question of permeability changes due to 2,4-D but is of interest as a comparison of the relative rates of uptake

TABLE IV

COMPARATIVE RATES OF SOLUTE UPTAKE BY CHLORELLA

SOLUTE	TEMPERATURE °C	MEAN CONCENTRATION IN CELLS μM/LITER	CONCENTRATION IN EXTERNAL SOLUTION μM/LITER	PERCENT EQUILIBRATION IN 20 MIN
Sucrose	1	6.89	6.50	106
Phosphate	1	2,686	10,000	27
Sulfate	1	2.26	7.07	32
Sodium	1	18.21	74.50	24
Sucrose	25	20.21	6.50	311
Mannitol	25	1.44	6.50	22
Phosphate	25	5,038	10,000	50
Sulfate	25	41.20	7.07	583
Sodium	25	24.20	74.50	32

of several organic and inorganic solutes under different temperature conditions. These data are summarized in table IV in terms of the calculated concentration within the cells after a 20-minute uptake from phosphate buffer at pH 5.5.

The substances investigated can be divided into 3 broad groups with respect to the time required to attain a concentration inside the cells equal to that in the external solution: 1) Mannitol at 20° C, sodium at 25° C and 1° C, phosphate at 1° C, and sulfate at 1° C all reach less than one-third of the external concentration within 20 minutes; 2) sucrose at 1° C comes to the same concentration inside the cell as in the external solution within 20 minutes on the basis of the mean of all determinations, although individual determinations vary from this mean (see fig 1); 3) sucrose at 25° C, and sulfate at 25° C exceed the external concentration in 20 minutes, presumably as a result of metabolic conversion or accumulation within the cell.

DISCUSSION AND CONCLUSIONS

Changes in permeability or solute uptake have been shown to result from auxin application to plants (1, 2, 5, 6, 7, 8, 10, 13, 14) and are thought to be of importance in some responses to auxins (7). It seems possible that a number of auxin effects may be due, partly at least, to changes in cell permeability to solutes or water or both. In addition to responses such as growth, cell elongation and changes in transpiration of leaves or fruits (7, 14), which may obviously involve permeability changes, other auxin effects on plants may also be caused by permeability changes. The inhibition of photosynthesis by 2,4-D (4, 16) might result from a decrease in the permeability of the cell to carbonate. The mobilization of reserves and polarization of solute translocation in plants may occur through local increases in permeability. If the semipermeable membrane of an actively metabolizing cell were altered so that solutes might enter the cell more readily, the result would be an effective increase in the supply of foods to that cell, since the rapid utilization of materials entering the

cell would maintain an inward gradient and prevent an equal increase in the rate of loss through outward diffusion.

The increase in solute uptake due to low concentrations of 2,4-D reported here is relatively small, although statistically significant. The low temperatures used probably tend to minimize increases or decreases in the rate of sucrose uptake since the free diffusion of sucrose may itself be partially limiting the uptake process under these conditions. With an environment more nearly normal for the growth of plants, the metabolic use of sucrose would proceed at a rate such that an increased permeability would result in a faster rate of sucrose supply and thus accentuate the effect of 2,4-D on the amount of labeled carbon from sucrose found in the cells, independent of any effect of 2,4-D on utilization of sucrose in the cells. Some support for this view may be obtained from the fact that at 25° C we have found a maximum increase of C^{14} in Chlorella cells, due to treatment with low concentrations of 2,4-D, of as much as 30 %, while at 1° C the increase amounts to only about 5 %.

Larger increases in permeability measured by plasmolysis-deplasmolysis time have been reported with indoleacetic acid treatments (5, 6, 8), although Ordin and Bonner (12) found that "optimal" (5 mg/liter) IAA had no effect on the diffusion of DHO into Avena coleoptile sections.

Since solute concentrations, temperatures and relative uptake rates were variable in these experiments, direct comparisons of the effect of 2,4-D on the uptake of various solutes cannot be made. However, if the amount of a solute found in the cells at a given concentration of 2,4-D is converted to a percentage of the amount found under the same conditions but in the absence of 2,4-D, it is possible to calculate regression equations for each solute independently and to compare the slopes for each line statistically. When this is done, the lines of best fit for the solute uptake experiments are found to be as follows: sucrose, $Y = 94.0 + 10.0\ X - 2.8\ X^2$; mannitol, $Y = 92.9 + 12.5\ X - 2.8\ X^2$; phosphate, $Y = 81.6 + 19.6\ X - 2.6\ X^2$; sulfate, $Y = 103.0 + 1.4\ X$; sodium $Y = 96.7 + 1.2\ X$.

Statistical analysis indicates that the linear component ($+ BX$) of the equations for sucrose, mannitol, and phosphate uptake is significantly different from zero at the 1 % level of confidence. The linear components for sulfate and sodium uptake do not differ significantly from zero. Comparison of the positive linear slope components indicates that sucrose and mannitol do not differ significantly, but that the slope for phosphate is significantly greater than that for either sucrose or mannitol at the 1 % level. The quadratic component ($- CX^2$) is statistically identical for sucrose, mannitol, and phosphate. No significant quadratic component exists for sulfate or sodium uptake.

From this analysis it is apparent that our samples include three populations of solutes with respect to the influence of 2,4-D on their uptake by Chlorella. The entry of sucrose and mannitol is increased significantly by 2,4-D at low concentrations, and this increase is overcome by the inhibitory effect of the quadratic component with an increase in concentration. The uptake of sodium and sulfate is not affected by 2,4-D under the conditions used for these permeability studies.

It should be repeated that these differences in uptake of various solutes as a result of 2,4-D treatment are being interpreted in terms of 2,4-D effects on permeability only in a relative sense. In spite of efforts to minimize its effect there still remains an undetermined fraction of the total uptake which is metabolic in nature and results in the conversion of the entering solute to other materials or causes an active accumulation of the solute over the amount which might enter by passive diffusion. We feel that this metabolic fraction is a small percentage of the whole and that the results obtained are an indication of 2,4-D influences on the rates of passage of several solutes through living membranes of Chlorella cells probably through alterations in the structure or composition of those membranes.

The mechanism by which 2,4-D brings about the changes in Chlorella permeability reported here remains obscure. The effect appears to be exerted only on permeability to uncharged particles, since only sucrose, mannitol, and phosphate, about 4 % of which exists as the undissociated molecular form at pH 5.5, demonstrated differences in rate of uptake as a result of 2,4-D treatment. The differences found between phosphate and sucrose or mannitol may be related to the penetration of phosphate anions which may occur by a different mechanism. It seems probable that phosphate would penetrate the cells most readily in the molecular form but some fraction of the total entering would be contributed by the anion (15). Glucose, water, glycerine and urea have been shown (7, 8) to be influenced in their uptake by auxin treatment in a fashion similar to that found here for sucrose, mannitol and phosphate. The highly charged Na^+ and SO_4^{--} ions were neither accelerated nor impeded in their passage into the cell by the presence of 2,4-D which may also indicate a different mechanism of entry for these particles. Masuda (8) obtained similar results when he found that 10^{-6} M IAA increased the permeability of onion scale epidermal cells to glycerine more than to urea, which may also indicate that highly polar particles are less affected by auxin-induced changes in permeability.

These facts may indicate that auxins are acting primarily on those properties of the membrane which permit passage of uncharged particles. If these properties are assumed to be associated with the structural configuration of the lipid phase of the membrane (3), then one possibility might be that auxins become a part of the membrane and in low concentrations result in an "opening" of the membrane structure. This might occur by solubilization of the lipophilic portion of the auxin molecule in the lipid phase of the membrane, with the charged carboxyl group oriented so that it could displace adjacent molecules electrostatically, opening "holes" in the membrane which provide a more ready entry for solute molecules.

The decrease in cell permeability with higher auxin concentrations might then result from a change in the composition of the membrane due to the incorporation of large numbers of auxin molecules and a resultant packing of the lipid membrane molecules and thus a decrease in the area receptive to solute diffusion. This hypothesis receives some support from the fact that the inhibitory component of the equations describing the influence of 2,4-D on sucrose, mannitol, and phosphate uptake is proportional to the second power of the 2,4-D concentration in the external medium, which could indicate that the inhibition results from a decrease in the area of the membrane across which the solutes could pass.

Other mechanisms of auxin action on cell permeability seem possible, although the present evidence is not sufficient to lend strong support to any particular hypothesis. The suggestion that auxin effects on permeability reside not in the membrane, but in the protoplast as a whole, which has been advanced by Meinl and Guttenberg (9, 10), mainly on the basis that gelatin and agar gels exhibit changes in physical properties such as swelling, viscosity, and permeability to water on treatment with indoleacetic acid, does not appear to be supported by our data, although the possibility is not eliminated.

Summary

Cells of *Chlorella pyrenoidosa* in concentrations of 2,4-D ranging from 10^{-7} to 10^{-2} M undergo pronounced alterations in permeability to some solute molecules. The uptake of sucrose-C^{14} and of mannitol-C^{14} is affected in similar fashion. With low concentrations of 2,4-D the uptake is greater in treated cells than in untreated ones, while an increase in 2,4-D concentration results in a progressive decrease in permeability, as indicated by inhibition of solute uptake.

Uptake of $P^{32}O_4$ by Chlorella is increased more by low 2,4-D concentrations than is that of the carbohydrates, while increased concentrations have less inhibitory effect on phosphate uptake.

Uptake of $S^{35}O_4$ and $Na^{22}Cl$ by Chlorella was not affected by 2,4-D under the conditions of this study.

The decrease in cell permeability in the presence of high 2,4-D concentrations was not due to the death of the cells.

Information is given on relative rates of uptake for the solutes studied under different temperature conditions.

The authors wish to express their gratitude to Dr. Ross C. Bean, who prepared the C^{14}-labeled sucrose and mannitol, and to Dr. Morris J. Garber, for his assistance in interpretation of certain statistical computations.

Literature Cited

1. Baas Becking, L. G. M. and Everson, R. G. Tissue tensions in *Bryophyllum calycinum* Salisbury. Australian Jour. Biol. Sci. 6: 347–368. 1953.
2. Brauner, L. and Hasman, M. Weitere Untersuchungen über den Wirkungsmechanismus des Heteroauxins bei der Wasseraufnahme von Pflanzenparenchymen. Protoplasma 41: 302–326. 1952.
3. Davson, H. and Danielli, J. F. The Permeability of Natural Membranes. Macmillan, New York 1943.
4. Erickson, L. C., Wedding, R. T. and Brannaman, B. L. Influence of pH on 2,4-dichlorophenoxyacetic acid and acetic acid activity in Chlorella. Plant Physiol. 30: 69–74. 1955.
5. Guttenberg, H. von and Beythien, Anna. über den Einfluss von Wirkstoffen auf die Wasserpermeabilitat des Protoplasmas. Planta 40: 36–69. 1951.
6. Guttenberg, H. von Meinl, G. Über den Einfluss von Wirkstoffen auf die Wasserpermeabilitat des Protoplasmas. II. Über den einfluss des pH-wertes und der Temperatur auf die durch Heteroauxin Bedingten Veranderungen der Wasserpermeabilitat. Planta 40: 431–442. 1952.
7. Kramer, P. J. Water relations of plant cells and tissues. Ann. Rev. Plant Physiol. 6: 253–272. 1955.
8. Masuda, Y. Über den Einfluss von Auxin auf die Stoffpermeabilität des Protoplasmas. I. Miteilung. Bot. Mag. (Tokyo) 66: 256–261. 1953.
9. Meinl, G. and Guttenberg, H. von. Über den Einfluss von Wirkstoffen auf die Permeabilitat des Protoplasmas. III. Der Einfluss des Heteroauxins auf Quellung, Viskositat und Wasserpermeabilitat von Gelatine und Agar. Planta 41: 167–179. 1952.
10. Meinl, G. and Guttenberg, H. von. Über den Einfluss von Wirkstoffen auf die Permeabilitat des Protoplasmas. IV. Über die Beeinflussung der Zuckerpermeabilitat von Protoplasten durch Heteroauxin. Planta 41: 179–189. 1952.
11. Ordin, L., Applewhite, R. H. and Bonner, J. Auxin-induced water uptake by Avena coleoptile sections. Plant Physiol. 31: 44–53. 1956.
12. Ordin, L. and Bonner, J. Permeability of Avena coleoptile sections to water measured by diffusion of deuterium hydroxide. Plant Physiol. 31: 53–57. 1956.
13. Reinhold, L. and Powell, R. G. The stimulatory effect of indole-3-acetic acid on the uptake of amino acids by tissue of *Helianthus annus*. Jour. Exptl. Bot. 9: 82–96. 1958.
14. Stewart, W. S., Palmer, J. E. and Hield, H. Z. Packing-house experiments on the use of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid to increase storage life of lemons. Proc. Amer. Soc. Hort. Sci. 59: 327–334. 1952.
15. Wedding, R. T. and Erickson, L. C. The role of pH in the permeability of Chlorella to 2,4-D. Plant Physiol. 32: 503–512. 1957.
16. Wedding, R. T., Erickson, L. C. and Brannaman, B. L. Effect of 2,4-dichlorophenoxyacetic acid on photosynthesis and respiration. Plant Physiol. 29: 64–69. 1954.

EFFECTS OF ATMOSPHERIC FLUORIDES AND VARIOUS TYPES OF INJURY ON THE RESPIRATION OF LEAF TISSUE[1]

A. C. HILL, M. R. PACK, L. G. TRANSTRUM AND W. S. WINTERS

AGRICULTURAL DIVISION—GENEVA WORKS, COLUMBIA-GENEVA STEEL DIVISION, UNITED STATES STEEL CORPORATION, PROVO, UTAH

The possibility of injury to vegetation by fluorides found in the atmosphere of certain urban and industrial areas has attracted considerable attention. At sufficiently high fluoride concentrations, varying with the sensitivity of the plant under consideration, a characteristic leaf necrosis is produced. Some investigators (13, 22, 24) have postulated that at lower concentrations atmospheric fluorides may have an adverse effect on certain physiological processes, but there are few data to support this contention. Certain biochemical studies have indicated that fluorides can affect respiration. Warburg and Christian (26) showed that fluoride inhibits the activity of enolase from yeast, and Miller (19) found a similar inhibition of enolase from pea seed. Several investigators have reported reductions in the respiration of plant tissues treated with fluoride solutions (3, 4, 7, 12, 15), and the fact that this reduction may be prevented by adding pyruvate indicates that it may be the result of an inhibition of enolase (3, 4, 15). There have been few attempts to determine if atmospheric fluorides may have a similar effect on growing plants. From the effects of spraying lime on the foliage of peach and prune trees, growing in an area where atmospheric fluorides were suspected of being present, McNulty and Newman (16) concluded that atmospheric fluorides inhibit respiration. In a later study (17), they found a higher rate of respiration in leaves of bush beans and gladioli fumigated with relatively high rates of hydrogen fluoride than in leaves of unfumigated control plants. It was concluded that the increase in respiration was caused by the presence of sub-lethal concentrations of fluoride in the leaf tissue, but the fluoride concentrations used caused intercostal chlorosis of the bean leaves and scorched the gladiolus leaf tips for a distance of 10 to 20 cm.

It has been known for some time that respiration increases in injured plant tissues (23). This has usually been attributed to facilitation of gaseous exchange at the wounded surface, but Whiteman and Schomer (27) found a definite wound stimulation of respiration in sweet potato roots. Audus (1) found that respiration of laurel leaf blades was increased by merely rubbing or bending them.

Investigations were conducted with a number of plant species to determine if atmospheric fluorides affect plant respiration. When an increase in respiration was observed in one species, further studies were made to determine whether this effect was caused by sub-lethal concentrations of fluoride in the tissue or whether it was dependent upon and directly related to the destruction of adjacent leaf tissue. The results of these investigations are presented here.

METHODS

Detailed studies were conducted in which plants grown in an atmosphere to which hydrogen fluoride was added were compared with others grown in a filtered atmosphere. As part of these studies, the effect of the fluoride treatments on oxygen uptake was determined on the following plants: Snow Princess gladioli (3 studies), Red Charm gladioli, Moscow tomatoes, Loren Blood tomatoes (2 studies), Marshall strawberries (3 studies), Utah 10-B celery (3 studies), Chinese apricots, Italian prunes (2 studies), and Bonneville barley. In celery studies II and III, the plants were grown in the field and covered with portable Vinylite chambers during the fluoride treatments. The other crops were grown in greenhouses, specially designed for controlling atmospheric fluorides. The facilities and techniques used for these controlled-atmosphere studies have been described elsewhere (10, 11). All environmental conditions within chambers or greenhouses being compared were kept as nearly the same as possible, except the fluoride treatments. Particular attention was given to maintaining the environment within the enclosures comparable to the natural plant habitat, since an atypical response to fluorides may occur under abnormal enviromental conditions.

The plants were grown in 3- by 5-foot plots of a uniform soil mix in the greenhouses, and the plantings being compared were grown in the same position in each greenhouse to avoid positional effects. Six trees were grown in each plot. With the other species the number of plants per plot ranged from 6 for tomatoes to 42 for some of the gladiolus studies and 125 to 150 for the barley. Chambers were placed over the individual plots during the fluoride treatments, except in the Snow Princess gladiolus I, Chinese apricot, Italian prune I and Bonneville barley studies. Air filtered through a lime-treated orlon baghouse system to remove ambient fluorides was circulated through each greenhouse at approximately 2 changes per minute. The filter system was very effective, removing about 98 % of the fluoride from the incoming air. The air was cooled in summer and heated in winter by drawing it through finned coils through which cold or hot water was circulated. The temperature, humidity, and light were nearly identical within the different

[1] Received June 12, 1958.

greenhouses and, during the regular growing season, differed very little from conditions in the field outside. One greenhouse or chamber was always maintained as a control, while hydrogen fluoride was introduced into the incoming air stream entering another. Different fluoride concentrations were used for different plant species, depending upon their susceptibility to fluoride injury. Several preliminary studies had been conducted using ambient and filtered air to determine if the fluorides in the local industrial plant atmosphere had any effect on plant respiration. No effect was observed, so in the studies with hydrogen fluoride reported here somewhat higher concentrations were used to determine if atmospheric fluorides might affect respiration if sufficiently high concentrations were used. The levels were fluctuated from day to day to simulate the variations encountered in the field and were generally increased as each study progressed to determine if an effect could be produced at a higher level.

Respiration was measured by determining the rate of oxygen uptake of leaf discs by the method of Umbreit et al (25), using the modifications suggested by Klinker (14). The leaves that appeared to have recently reached full development were used for these measurements. Three sets of 9 to 12 leaves were collected at random from each plot. The respiration measurements were made in triplicate on each set, giving 9 determinations for each treatment every time measurements were made. Leaf discs were cut with an 8-mm cork borer from 2 to 5 cm below any necrosis on the gladiolus leaves. With the other plants, if any necrotic leaves were present they were avoided for the respiration measurements, and the leaf discs were cut from midway between the midrib and the leaf margin. In most of the studies, respiration was measured at weekly intervals, beginning prior to the fluoride treatments and continuing throughout the studies.

The leaf samples were analyzed for fluoride by a modification of the method of Willard and Winter (28), after being rinsed with distilled water. Air in the environmental chambers was sampled continuously and analyzed for fluoride by modifications of the methods of Nielsen (20) and Nielsen and Dangerfield (21).

To study the effects of injury on the respiration rate of the adjacent, uninjured tissue, Snow Princess gladioli were grown in the control greenhouse. When the plants reached the 5- to 6-leaf stage of growth, randomly-selected 3rd and 4th leaves on the intact plants were injured for a distance of 20 cm from the tip by the following methods: scalding by dipping in hot water, burning with a hot iron, and crushing with a hammer while held against a board. Three days after the injury was inflicted, oxygen uptake was measured on leaf discs from immediately adjacent to, 5 cm below, and 15 cm below the injured areas. In this and subsequent injury studies, the rate of oxygen uptake of leaf discs from the injured leaves was compared to that of discs from corresponding positions on uninjured leaves, grown at the same time and under the same conditions except for the injury treatments. The measurements were made in triplicate on each of 3 sets of leaves, making 9 determinations for each comparison. There were 9 leaves to a set.

For comparison with the physically injured gladioli, another planting was subjected continuously to an atmosphere containing hydrogen fluoride at an average fluoride concentration of 0.35 $\mu g/m^3$.[2] When the gladiolus plants reached the 5- to 6-leaf stage of growth, many of the 3rd and 4th leaves had scorched for a distance of about 20 cm from the tip. Leaf samples were collected, and oxygen uptake measurements were made on leaf discs taken at the same distances from the injured areas as with the physically injured leaves. Another comparison was made in which 20 cm of the tips were cut from leaves on gladiolus plants. In this test respiration measurements were made adjacent to the cut edge only.

To determine if different amounts of injury affect respiration to a different extent, Snow Princess gladioli that had been grown in a greenhouse with filtered air were injured by dipping the leaves in hot water for distances of 10, 20, and 30 cm from the tip. Three days later, oxygen uptake was measured immediately below and 10 cm below the injury.

Results

The data in table I summarizes the controlled atmospheric fluoride studies with various plant species. These plants were subjected to continuous hydrogen fluoride treatments for several weeks. The fluoride levels given are averages for the entire treatment period. Since it was difficult to replicate the treatments because of the limited facilities, several studies were conducted with most of the plants. Higher fluoride levels were generally used in the later studies in an attempt to produce an effect. The data given for respiration and fluoride content of leaves are averages of the last 2 weekly sets of measurements, except for the gladioli where the final measurements are related to given amounts of necrosis and 4 studies where determinations were not made every week. These data represent the period when the greatest effect of the treatments might be expected.

None of the fluoride treatments had a significant effect on respiration, except in the 2 varieties of gladioli where respiration was higher in the fluoride-treated plants. When all studies of any one crop are considered it becomes further apparent that the nonsignificant differences are independent of the treatments. The hydrogen fluoride treatments were high enough to cause some leaf necrosis on all plants except celery and barley, and to produce high concentrations of fluoride in the tissue.

Gladioli are very sensitive to fluoride and developed considerable necrosis under the fluoride treatments.

[2] All atmospheric analyses are expressed in μg fluoride per m^3 air at 29.92 in Hg pressure and 60° F temperature. For comparison with other units of expression 1 $\mu g/m^3$ of fluoride is equal to 0.874 ppb (parts per billion) by weight of fluoride or to 1.33 ppb by volume of any gas containing 1 fluorine atom per molecule.

TABLE I

EFFECT OF HYDROGEN FLUORIDE ON THE FLUORIDE CONTENT AND RATE OF OXYGEN UPTAKE OF PLANT LEAVES

PLANT VARIETY	LENGTH OF TREATMENT	AVG F TREATMENT	F CONTENT OF WASHED LEAVES, DRY WT		OXYGEN UPTAKE, INCREASE OF TREATED ABOVE CONTROL	NECROSIS‡
			CONTROL	TREATED		
	days	*μg/m³*	*ppm*	*ppm*	%	%
Snow Princess gladiolus†	29	0.90	6	38	50.0**	30
Snow Princess gladiolus II†	40	0.97	9	58	40.0**	45
Snow Princess gladiolus III†	40	0.76	9	65	39.0**	46
Red Charm gladiolus†	28	1.50	11	77	27.0*	20
Moscow tomato	26	5.70	24	500	—1.5	
Loran Blood tomato I	26	9.00	18	820	—14.0	
Loran Blood tomato II	21	47.00	18	2400	—6.0	
Marshall strawberry I	63	2.20	10	160	12.0	
Marshall strawberry II†	23	5.60	10	470	0.7	
Marshall strawberry III	49	6.90	14	670	—9.4	
Utah 10-B celery I†	41	1.10	9	140	1.3	
Utah 10-B celery II	27	11.00	58††	2200	11.0	
Utah 10-B celery III	22	25.00	50††	2500	4.3	
Chinese apricot	44	1.10	8	58	—6.2	
Italian prune I	35	1.10	4	62	—1.6	
Italian prune II†	53	3.00	6	510	1.3	
Bonneville Barley†	73	0.32	4	130	—5.9	

* Significant at 5 % probability level.
** Significant at 1 % probability level.
† F content of leaves and oxygen uptake for these crops are final determinations only. Data for other crops are average of last 2 weekly determinations.
†† Control treatments of celery studies II and III exposed to unfiltered ambient air.
‡ Measurements of respiration made on necrotic leaves of gladioli and on uninjured leaves of other plants.

Since they were the only plants in which respiration was affected and the effect was not observed until the necrosis developed, it appeared that this effect might be related to the leaf necrosis rather than the fluoride in the tissue. The amount of fluoride in the atmosphere or in the leaves had no apparent influence on respiration.

Several studies were conducted to clarify the relationship of respiration to injury of gladiolus leaves. Table II summarizes studies in which Snow Princess gladiolus leaves were injured by 3 different physical methods and by hydrogen fluoride. The leaves were injured for 20 cm from the tip. The increase in respiration rate was highly significant adjacent to the necrotic area with all types of injury tested, and the fluoride-injured leaves showed a highly significant increase in respiration 5 cm from the injury. The increase was not significant at 5 cm for any of the other types of injury or at 15 cm for any including fluoride injury, although there was a tendency toward a higher rate of respiration in all of the measurements made on injured leaves. These data also show that the effect of the injury on the respiration rate was confined largely to the tissue near the injury. When 20 cm of the tips were cut from gladiolus leaves, the respiration rate of the tissue adjacent to the cut averaged 2.8 % lower than the controls, a difference that was not significant. It appears, therefore, that an increase in respiration occurs only when injured tissue is left attached, indicating that it may be caused by some substance that is transferred from the injured tissue.

TABLE II

RESPIRATION RATE OF SNOW PRINCESS GLADIOLUS LEAVES AS RELATED TO DIFFERENT TYPES OF INJURY AND DIFFERENT DISTANCES FROM THE INJURY

DISTANCE BELOW INJURY	OXYGEN UPTAKE INCREASE ABOVE CONTROL, %			
	CRUSHED	SCALDED	DRY HEAT	FLUORIDE
Adjacent	23.1	42.4	30.7	53.0
5 cm	0.5	7.0	3.8	20.7
15 cm	6.7	3.4	7.1	10.8
L.S.D.* 5 %		14.3		12.7
1 %		19.6		18.1

*Least Significant Difference.

The fluoride-induced necrosis produced a greater increase in respiration than that produced by the physical methods at 5 cm, but not greater than that produced by scalding adjacent to the injury. There are two reasons why a greater increase might be expected with fluoride injury. First, the margins of the fluoride injury are very irregular, usually extending further down the margins than in the center of the leaf, as compared to the straight margins of the physical injury. As a result, discs taken from adjacent to the necrosis on the fluoride-injured leaves had a smaller

TABLE III

RESPIRATION RATE OF SNOW PRINCESS GLADIOLUS LEAF DISCS AS RELATED TO DIFFERENT AMOUNTS OF INJURY BY SCALDING

DISTANCE BELOW INJURY		OXYGEN UPTAKE INCREASE ABOVE CONTROL, %		
		10 CM INJURY	20 CM INJURY	30 CM INJURY
Adjacent		14.3	25.7	28.8
10 cm		—6.8	—2.4	5.0
L.S.D.*	5 %		15.3	
	1 %		21.2	

*Least Significant Difference.

mean distance from necrotic tissue. Second, a 3-day period elapsed between the time the injury was inflicted by the physical means and the time of the respiration determinations, whereas the fluoride induced injury was developing continually. If a substance is translocated downward from the injured zone, it probably occurs in a higher concentration during or soon after the death of the tissue, which could well cause the differences found.

Scalding was used to determine if the response of respiration is proportional to the amount of injury (table III). When only 10 cm of the leaf tip were injured, the increase in respiration rate of the adjacent tissue fell slightly short of significance. With 20 or 30 cm of injury, the increase over the control was highly significant adjacent to the injury. Respiration was not significantly affected 10 cm from the injury by any of the treatments. The respiration rate of the tissue adjacent to the injury had a tendency to increase with greater amounts of injury, but the increase was not quite significant at the 5 % level for the range of injury used.

DISCUSSION

Interpreting the data in table I in light of the results of the studies with physical injury, it may be concluded that the only effect of atmospheric fluorides on the respiration rate of the plants was indirect, contingent upon the injury of some of the adjacent tissue. Only in those leaves with necrotic tissue was oxygen uptake significantly affected. The amount of fluoride in the tissue did not appear to be a significant factor. In the other species, in which uninjured leaves were used, the respiration rate was unaffected in spite of the accumulation of very high concentrations of fluoride.

The only other reported studies of the effects of atmospheric fluorides on plant respiration are those by McNulty and Newman (16, 17), and the results have been conflicting. The conclusion from the lime spray study (16) that fluoride causes a reduction in respiration is somewhat weakened by the lack of analyses to verify the presence of fluoride. The later study, in which an increase in respiration of bush beans and gladioli developed after they were fumigated with sufficiently high concentrations of hydrogen fluoride to produce visible injury (17), appears to be in agreement with the data reported here.

The difference in response of respiration to solution and atmospheric fluoride treatments might be due to differences in the fluoride concentrations at the reaction site in the cytoplasm under the two types of exposure. Bonner and Thimann (5) reported that 0.003 M sodium fluoride did not affect the respiration of etiolated pea stems and oat coleoptiles, and Christiansen et al (8) found that the inhibition of respiration of etiolated pea stems by 0.005 M sodium fluoride disappeared after 12 hours. This indicates that sodium fluoride solutions of approximately 0.005 M, or about 100 ppm fluoride, are required to affect measurements of over-all respiration. Since little is known about the relative concentrations of fluoride in different parts of the cell, it is difficult to make a direct comparison between the solution and atmospheric studies, but the fact that relatively high fluoride concentrations accumulated in the tissue of plants subjected to atmospheric fluorides with no measurable effect on oxygen uptake indicates that much of the fluoride must have been inactivated somewhere within the plant. Possible sites of such inactivation include the cell wall and the vacuole. Inactivation within the vacuole appears to permit the most satisfactory explanation of the difference in response to solution and atmospheric fluorides. It has been shown that some inorganic ions tend to accumulate in the vacuole in concentrations as much as 100 times those in the circumambient solution (18). Fluoride may accumulate similarly, and much of it could precipitate within the vacuole if the concentration exceeds the solubility of the salts of such cations as calcium and aluminum.

The fluoride concentrations required to inhibit respiration in solution studies are very high in comparison to the atmospheric fluoride concentrations used in the studies reported here, which undoubtedly results in more rapid absorption of fluoride from the solutions. Briggs and Robertson (6) contend that a somewhat uniform solute concentration exists throughout the cytoplasm and extracellular solution. If this is true, the concentration of fluoride in the cytoplasm might tend to approach the same order of magnitude as in a solution in which the tissue is immersed. Likewise, in atmospheric studies the fluoride concentration in the extracellular solution and cytoplasm could be in a steady state with the concentration in the atmosphere and at some site of accumulation such as the vacuole. Because of the affinity of fluoride for water, the concentration in the cytoplasm may be considerably higher than in the atmosphere to which the plants are exposed, but with a reasonable rate of movement into the vacuole the concentration in the cytoplasm should not build up to more than a few ppm, even when atmospheric concentrations are high.

If enolase activity or other processes that might be inhibited by fluorides are measured on extracts or tissue homogenates, the results may be very mislead-

ing, because any restrictions on the contact of the fluoride with the enzyme systems should be largely removed by macerating the tissue. Inhibitions might be indicated even though no such inhibitions occurred in the intact plant. Enolase activity was not measured in these studies, but if a reduction in activity occurred under the fluoride treatments it apparently was not reflected in oxygen uptake. It is possible that enolase activity might be reduced appreciably before it becomes the limiting factor in glycolysis. Another possibility is that the Warburg-Dickens direct oxidation pathway, which appears to be of considerable importance in mature tissues (2, 9), might partially compensate for any inhibition of enolase.

In relation to air pollution, the major interest is in determining whether or not there is an effect on vegetation growing under the atmospheric fluoride concentrations that occur around industry. Usually a major part of the fluoride in the atmosphere is in the form of rather insoluble solid particles that have little or no effect on plants. Since hydrogen fluoride was used in these studies, the hydrogen fluoride concentration or its phytotoxic equivalent in the air, rather than the total fluoride concentration, must be considered when attempting to apply these results to the field. Comparison of hydrogen fluoride injury to sensitive plants in long term fumigation studies with injury observed on the same plants growing in the field around fluoride emitting industries indicate that the average hydrogen fluoride phytotoxic equivalent in the vicinity of modern industries is seldom more than a few tenths of a $\mu g/m^3$. Chemical analysis of the air for gaseous fluorides indicates a rather good correlation between the gaseous fluoride concentration and the phytotoxic equivalent and further substantiates this conclusion. Since these studies showed no direct effect of fluoride on respiration of a variety of plants when much higher fluoride concentrations were used, it is very unlikely that fluorides in the atmosphere ever have a direct effect on the respiration of vegetation growing in the field.

Summary

Seven species of plants were grown under various atmospheric hydrogen fluoride treatments for several weeks, and measurements were made of the respiration rate of leaf discs and the fluoride content of the leaf tissue. No direct effect of the fluoride on respiration was detected, even when very high fluoride concentrations were used. An increase in oxygen uptake, which appeared to be related directly to the development of leaf necrosis, was found in 2 varieties of gladioli.

Injury of gladiolus leaves by scalding, crushing, and burning with a hot iron caused an increase in the respiration rate of the adjacent tissue comparable to that found when leaf scorch was produced by hydrogen fluoride in the atmosphere. Removal of part of a leaf by cutting did not affect the respiration of the remaining leaf tissue. The increase in respiration was greatest near the injured tissue and was somewhat proportional to the amount of injury produced.

An explanation is offered for the apparent disagreement of the results with the reported inhibitions of respiration and enolase activity by fluoride solutions.

The data indicate that it is very unlikely that fluorides in the atmosphere of urban or industrial areas ever affect the respiration of vegetation, at least not without first producing much more obvious effects such as the development of severe leaf necrosis.

The advice and assistance of Dr. Leo P. Vernon, Brigham Young University Chemistry Department, in the initial stages of these investigations and the technical assistance of W. S. Gardner, L. K. Kirk, A. Holloman, Jr., R. J. Downs, and K. L. Hanks in growing the plants and performing the determinations are gratefully acknowledged.

Literature Cited

1. Audus, L. J. Mechanical stimulation and respiration rate in cherry laurel. New Phytologist 34: 386–402. 1935.
2. Beevers, H. and Gibbs, M. The direct oxidation pathway in plant respiration. Plant Physiol. 29: 322–324. 1954.
3. Bonner, J. Biochemical mechanisms in the respiration of the Avena coleoptile. Arch. Biochem. 17: 311–326. 1948.
4. Bonner, J. and Wildman, S. G. Enzymatic mechanisms in the respiration of spinach leaves. Arch. Biochem. 10: 497–518. 1946.
5. Bonner, W. D., Jr. and Thimann, K. V. Studies on the growth and inhibition of isolated plant parts. III. The action of some inhibitors concerned with pyruvate metabolism. Amer. Jour. Bot. 37: 66–75. 1950.
6. Briggs, G. E. and Robertson, R. N. Apparent free space. Ann. Rev. Plant Physiol. 8: 11–30. 1957.
7. Christiansen, G. S. and Thimann, K. V. The metabolism of stem tissue during growth and its inhibition. II. Respiration and ether soluble material. Arch. Biochem. 26: 248–259. 1950.
8. Christiansen, G. S., Kunz, L. J., Bonner, W. D., Jr. and Thimann, K. V. The action of growth inhibitors on carbohydrate metabolism in the pea. Plant Physiol. 24: 178–181. 1949.
9. Gibbs, M. and Beevers, H. Glucose dissimilation in the higher plant. Effect of age of tissue. Plant Physiol. 30: 343–347. 1955.
10. Hill, A. C., Transtrum, L. G., Pack, M. R. and Holloman, A., Jr. Facilities and techniques for maintaining a controlled fluoride environment in vegetation studies. Jour. Air Pollution Control Assoc. (In press.)
11. Hill, A. C., Transtrum, L. G., Pack, M. R. and Winters, W. S. An investigation of the "hidden injury" theory of fluoride damage to plants. Agron. Jour. 50: 562–565. 1958.
12. James, W. O. Respiration of plants. Ann. Rev. Biochem. 15: 417–434. 1946.
13. Katz, M. and Shore, V. C. Air pollution damage to vegetation. Jour. Air Pollution Control Assoc. 5: 2–8. 1955.

14. KLINKER, J. E. A modification of the Warburg respirometer to measure the respiration rate of tomato leaf discs. Plant Physiol. 25: 354–355. 1950.
15. LATIES, G. G. The role of pyruvate in the aerobic respiration of barley roots. Arch. Biochem. 20: 284–299. 1949.
16. MCNULTY, I. B. and NEWMAN, D. W. The effects of a lime spray on the respiration rate and chlorophyll content of leaves exposed to a fluoride atmosphere. Proc. Utah Acad. Sci. 33: 73–79. 1956.
17. MCNULTY, I. B. and NEWMAN, D. W. Effects of atmospheric fluoride on the respiration rate of bush bean and gladiolus leaves. Plant Physiol. 32: 121–124. 1957.
18. MEYER, B. S. and ANDERSON, D. B. Plant Physiology. Pp. 462–464. D. Van Nostrand, New York 1952.
19. MILLER, G. W. Properties of enolase extracts from pea seed. Plant Physiol. 33: 199–206. 1958.
20. NIELSEN, H. M. Determination of microgram quantities of fluoride. Anal. Chem. 30: 1009–1011. 1958.
21. NIELSEN, J. P. and DANGERFIELD, A. D. Use of ion exchange resin for determination of atmospheric fluorides. A. M. A. Arch. Ind. Health 11: 61–65. 1955.
22. RECKENDORFER, P. Ein Beitrag zur Mikrochemie des Rauchschadens durch Fluor; Die Wanderung des Fluors im pflanzlichen Gewebe. I. Teil: Die unsichtbaren Schaden. Pflanzenschutz-Berichte 9: 33–55. 1952.
23. RICHARDS, H. M. The respiration of wounded plants. Ann. Bot. 10: 531-582. 1896.
24. THOMAS, M. D. The invisible injury theory of plant damage. Jour. Air Pollution Control Assoc. 5: 205–208. 1956.
25. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric Techniques and Tissue Metabolism. Burgess, Minneapolis 1945.
26. WARBURG, O. and CHRISTIAN, W. Isolierung und Kristallisation des Garungsferments Enolase. Biochem. Zeits. 310: 384–421. 1942.
27. WHITEMAN, T. M. and SCHOMER, H. A. Respiration and internal gas content of injured sweet-potato roots. Plant Physiol. 20: 171–182. 1945.
28. WILLARD, H. H. and WINTER, O. B. Volumetric method for determination of fluorine. Ind. Eng. Chem., Anal. Ed. 5: 7–10. 1933.

AN ANALYSIS OF AUXIN-GIBBERELLIN INTERACTION IN PEA STEM TISSUE[1,2]

ARTHUR W. GALSTON AND HAVA WARBURG
JOSIAH WILLARD GIBBS RESEARCH LABORATORY, DEPARTMENT OF BOTANY, YALE UNIVERSITY, NEW HAVEN, CONN.

It has previously been reported from various laboratories (1, 8, 10, 14, 18) that etiolated pea epicotyl sections are promoted in their growth both by indoleacetic acid (IAA) and by gibberellic acid (GA) supplied separately. When GA and IAA are supplied together in optimal concentrations, the total growth increment never exceeds and usually does not equal the sum of the separate growth increments (14). This lack of interaction between GA- and IAA-mediated growth favors the view that these systems are metabolically distinct and possibly entirely unrelated. In contrast with this view are the results of experiments with green pea sections and decapitated etiolated pea plants (2, 17) from which it appears that GA-induced growth is synergistic with and possibly completely dependent on an IAA or auxin-induced growth.

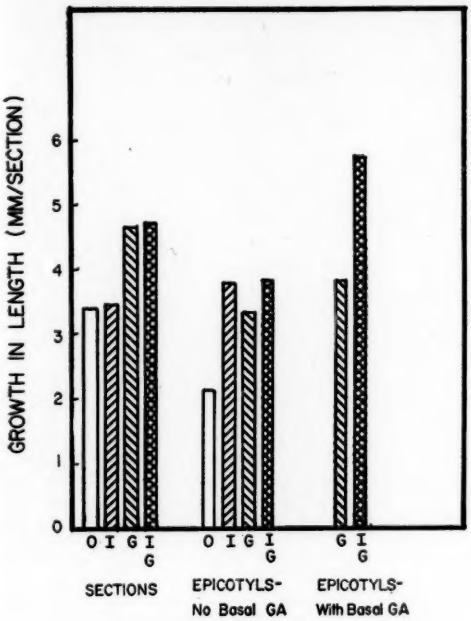
The present work contributes to a resolution of this apparent discrepancy by demonstrating that, in etiolated peas, purely additive responses to the two growth factors are obtained when short (5 mm long) epicotyl sections are excised and treated, but that synergistic interactions are obtained when longer epicotyl pieces are exposed, at different loci, to appropriate concentrations of the two substances. The results are interpreted as supporting the "three-factor" hypothesis recently advanced by Brian and Hemming (2) to explain the control of growth in stems.

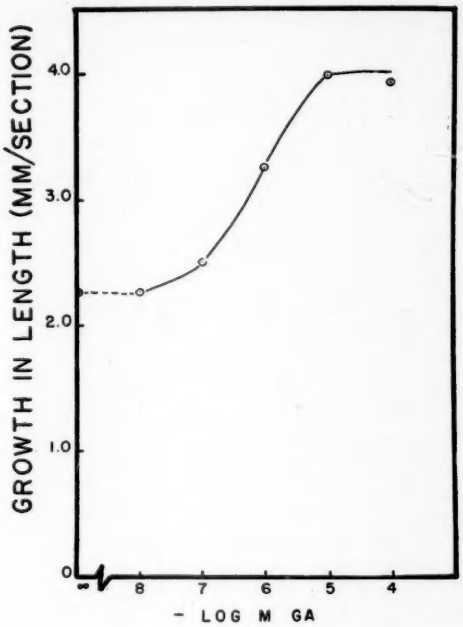
[1] Received June 23, 1958
[2] Research supported by the National Science Foundation under grant NSF G-2846

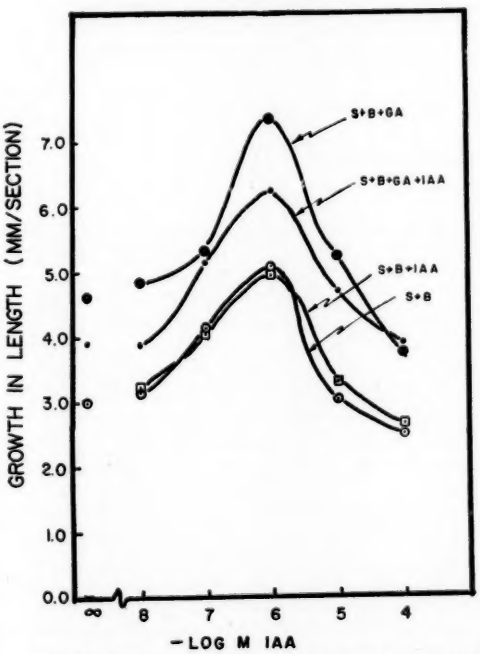
MATERIALS AND METHODS

Alaska peas (*Pisum sativum* L.) were obtained from Associated Seed Growers, Inc., New Haven, Connecticut. They were permitted to imbibe for 4 hours in tap water in darkness, and were then sown in polyethylene trays containing water-saturated vermiculite (Mica-Gro Type B) which had previously been washed in running water to remove an inhibitor of growth. They were grown in a darkened cabinet in a dark room maintained at 27° ± 1° C. The occasional illumination required for handling of the material was furnished by a dim green source found to be inactive both phototropically and photomorphogenically. This source consisted of a 15-watt Sylvania green fluorescent tube wrapped with 3 layers of green and 3 layers of amber duPont cellophane, and located about 30 cm from the working table area.

At the age of 7 days, the epicotyls were ready for harvest. The only seedlings selected for use in the growth experiments were those with recurved terminal buds and 3rd internodes between 20 and 40 mm long. Selected seedlings were severed slightly above the ground line with a razor, decapitated by a gentle stroking motion between thumb and forefinger, and trimmed at the base to a length of 100 mm. Such 100 mm long epicotyls were, in certain experiments, treat-







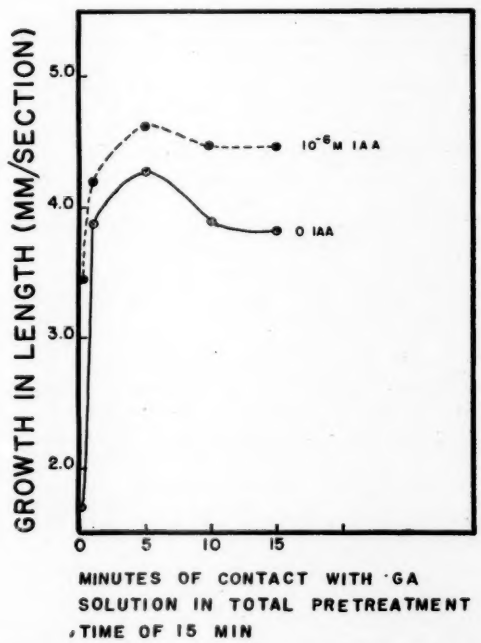

GROWTH IN LENGTH (MM/SECTION)
2.0 3.0 4.0 5.0
10⁻⁶M IAA
0 IAA
0 5 10 15
MINUTES OF CONTACT WITH GA SOLUTION IN TOTAL PRETREATMENT TIME OF 15 MIN

FIG. 1 (*upper left*). The effect of GA and IAA administered in various ways on the growth of excised pea epicotyl sections.

FIG. 2 (*upper right*). The relation between molarity of GA in the pretreatment solution and subsequent growth of excised sections.

FIG. 3 (*lower left*). Relation of the composition of the pretreatment medium to growth of sections in various concentrations of IAA. S + B = sucrose and buffer.

FIG. 4 (*lower right*). The effect of time of contact of epicotyls with GA on the subsequent growth of excised apical sections. Total pretreatment time 15 minutes.

ed basally with GA. Apical sections 5 mm long, equivalent to the S1 sections of Purves and Hillman (14), were obtained from the epicotyls by the use of a guillotine previously described (6). For the growth tests, 12 such sections were incubated in 10 ml of medium in 10 cm diameter Petri dishes for about 18 hours in the dark room. They were then measured to the nearest 0.1 mm under a dissecting microscope equipped with an ocular micrometer. Standard errors were computed for the means of all growth experiments. These values were almost uniformly below 5 % of the mean growth increment and, in all experiments reported in this paper, did not exceed 8 %. Occasional experiments in which the growth data were more variable than this were discounted entirely.

Fresh weights of the groups of sections were routinely obtained to the nearest milligram after gentle blotting. For certain critical experiments, weights of the individual groups were also obtained in the dark room prior to immersion in the growth medium. Although no weight data are presented in this paper, the results of such measurements confirm in all details the conclusions reached with length measurements.

The growth medium always contained 2 % sucrose and .02 M pH 6.1 KH_2PO_4-Na_2HPO_4 buffer (Fisher certified reagents) unless otherwise stated. IAA, obtained from the Nutritional Biochemical Company, was made up as a 10^{-3} M stock solution in an equivalent of $KHCO_3$, adjusted to pH 7.0 with KOH and stored in a refrigerator for no longer than 1 month. The GA, obtained from Dr. P. W. Brian of Imperial Chemical Industries, England, was similarly made up to a 10^{-3} M stock solution, and stored in the refrigerator for no longer than 1 month. The distilled water used in these experiments was drawn directly from the tap of the stainless steel delivery line. The conductivity of such line distilled water never exceeded 0.3 ppm NaCl equivalents, as measured with a Barnstead purity meter, model PM-2. The small quantities of chromium in such water were found not to influence the growth of the sections, since glass-redistilled water gave no improvement in growth.

Results

Growth Effects of GA Supplied Directly to Apical Sections or Basally to Epicotyls: GA and IAA were supplied in various ways to subapical sections of peas and the effects on growth noted. The varying procedures were as follows:

1.) Sections were excised, placed directly in growth media containing 10^{-6} M IAA, 10^{-4} M GA, neither, or both, in addition to sucrose and buffer. 2) 100 mm long epicotyls were pretreated for 30 minutes with their bases immersed in 10 ml of sucrose and buffer medium in a 50 ml beaker; sections were then excised and placed either in basal medium or medium supplemented by 10^{-6} M IAA, 10^{-4} M GA or both. 3) Like 2), but the pretreatment solution contained 10^{-4} M GA in addition to sucrose and buffer. In effect, such a procedure permits the separate calculation of the growth effects of GA and/or IAA, administered either directly to excised sections or to the epicotyl stick. Typical data from such an experiment are shown in figure 1.

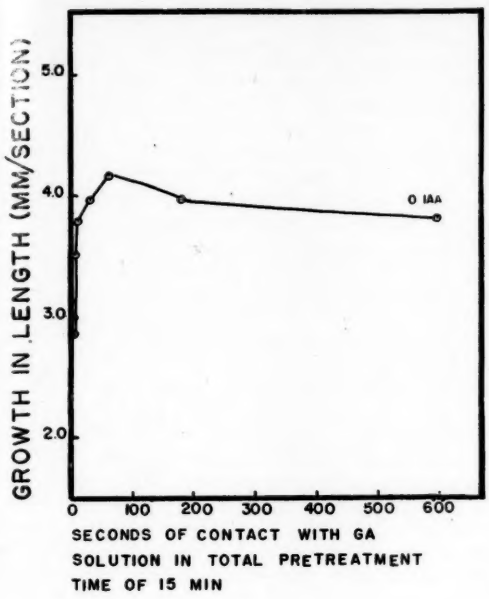
The following points should be noted: (a) Sections excised and placed directly in the growth medium are practically insensitive to 10^{-6} M IAA, but are very responsive to GA. Their response to GA and IAA supplied together does not exceed the expected additivity. These results conform well with those previously reported by others in this laboratory (14). (b) Sections derived from epicotyls pretreated basally with sucrose-buffer show a greatly depressed endogenous growth, a greatly heightened sensitivity to IAA and a slightly depressed response to GA. Growth in response to GA and IAA supplied together is far less than the expected additivity. These results can probably be interpreted as being the result of depletion, during the pretreatment, of growth substances from the region of the epicotyl subsequently excised and used for the growth study. (c) Sections derived from epicotyls pretreated basally with GA-containing solutions show significantly greater growth, when supplied with IAA, than any other sections. The combined effect of the IAA and GA treatment far exceeds the expectation of simple additivity, and may thus be considered synergistic.

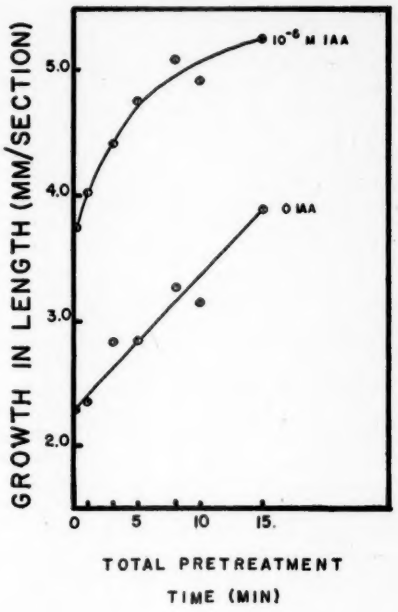
This synergism could be considered to result either from consequences of the passage of GA through the tissue on its way to the apex, or to the 30-minute time lag between the GA and IAA application. An experiment was thus performed in which the GA was supplied to excised sections for 30 minutes, after which time the sections were removed, thoroughly rinsed, and transferred to IAA-containing media. The growth of such sections was no greater than that of sections placed directly in GA and IAA.

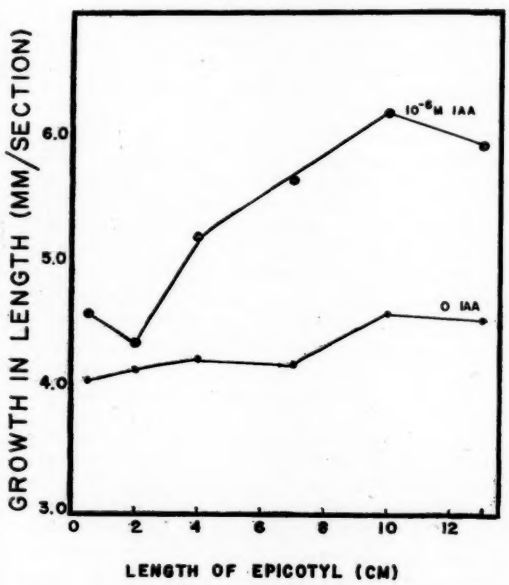
An analogous experiment was also performed in which the apical end of the epicotyl was dipped into GA during the pretreatment period, and the apical sections ultimately excised and placed in IAA-containing solutions. The growth under such circumstances was *less* than the growth of excised sections, and showed no synergistic interaction of IAA and GA. The synergism is thus not due to the temporal lag between GA and IAA application, and hence appears to result from some effect of GA application at the base of the epicotyl, about 100 mm away.

Relation of GA Concentration in the Pretreatment Solution to Subsequent Growth: Groups of epicotyls were treated for 30 minutes with sucrose and buffer solutions containing varying molarities of GA. At the conclusion of the pretreatment, sections were excised and placed in solutions containing sucrose and buffer. The growth results are shown in figure 2. There is a slight response to 10^{-7} M GA, half maximal response to 10^{-6} M, and optimal response in the range 10^{-5} to 10^{-4} M.

In another series of experiments, the interrelation between condition of pretreatment of the epicotyl and subsequent response of the section to a range of IAA concentrations was studied. From figure 3, it can be seen that pretreatment with 10^{-4} M GA raises the subsequent growth of the sections without altering the







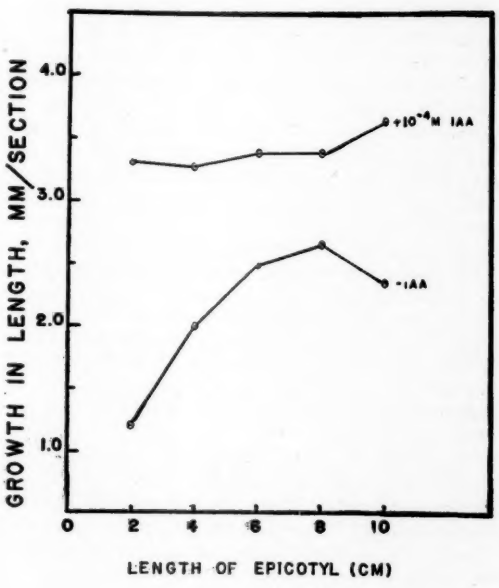


FIG. 5 (*upper left*). The effect of time of contact of epicotyls with GA on the subsequent growth of excised apical sections. Total pretreatment time 15 minutes.

FIG. 6 (*upper right*). Effect of total pretreatment time with GA on subsequent growth of excised sections.

FIG. 7 (*lower left*). The effect of length of epicotyl exposed to GA on subsequent growth of sections.

FIG. 8 (*lower right*). The effect of length of GA-treated green pea stems on the growth response of excised apical sections.

optimal IAA concentration; pretreatment with IAA produces a slight but significant depression of growth at some subsequent IAA levels; inclusion of IAA in the GA pretreatment medium results in a marked depression of the GA effect. Thus, GA and IAA appear to react synergistically when supplied at the different loci, but antagonistically when both are supplied basally. In another experiment, not presented here, it was found that the greater the GA concentration of the pretreatment solution, the greater is the growth of the sections at all IAA concentrations.

Relation Between Duration of Epicotyl Pretreatment with GA and Subsequent Growth of Sections: It became apparent, during the various experiments, that the arbitrary pretreatment time of 30 minutes and the arbitrary pretreatment solution volume of 10 ml per 12 epicotyls were much greater than necessary. Figure 4 shows the result of an experiment in which 12 epicotyls were immersed in 1 ml of solution (sucrose + buffer + 10^{-4} M GA) in a 10 ml beaker for varying numbers of minutes. In all cases, the epicotyls were transferred, after exposure to GA, to another beaker containing sucrose and buffer only, and the apical sections uniformly excised after 15 minutes had elapsed since the 1st basal exposure to GA. Figure 5 shows a subsequent similar experiment in which the exposure time to GA was reduced to seconds.

From these graphs, it is clear that as little as 1 to 3 minutes of basal exposure to 10^{-4} M GA suffices to elicit the maximum growth response, if the apical 5 mm sections are excised from the 100 mm epicotyls a total of 15 minutes after their 1st exposure to GA. It also appears that as little as 3 seconds of exposure to GA produces a marked effect on subsequent growth.

To determine the apparent maximum rate of transport of the GA or its induced effect up the epicotyl, an additional experiment was performed in which the epicotyls were immersed for varying periods of time in 10^{-4} M GA and the sections excised without further delay. From figure 6 it is clear that a significant effect of basally-applied GA is apparent at the apex after 3 to 5 minutes, and that more and more effect is apparent at the apex as the total time of exposure increases, with some evidence of a plateau between 10 to 15 minutes in the + IAA cultures. This implies a translocation rate of GA or a GA-induced change as rapid as 20 to 33 mm/min.

Relation Between Length of Epicotyl Treated with GA and its Subsequent Growth Effect: The previous data indicate that GA or a GA-induced change, moving up the epicotyl, produces some effect which leads to greatly enhanced growth in the presence of IAA. In other experiments, it became apparent that the effect of GA is directly proportional to the length of epicotyl employed in the experiment. Epicotyls of various lengths from 5 to 100 mm were placed with their basal ends in a solution of 10^{-4} M GA in sucrose-buffer. After a standard exposure time of 15 minutes, 5 mm sections were excised from the apex, and placed in 10^{-4} M IAA and sucrose-buffer. The results, shown in figure 7, demonstrate that the effect of GA is greater the longer the epicotyl, provided the sections are grown in the presence of IAA. There is also a much smaller but significant effect of length of epicotyl on growth of sections in the absence of IAA.

Gibberellin-auxin Interactions In Green Pea Stems: Brian and Hemming (2) have shown convincingly that sub-apical stem sections derived from light-grown peas react to GA only in the presence of an auxin. The GA-IAA interaction in green sections, unlike that in etiolated sections, is clearly synergistic. It occurred to us, therefore, to repeat our experiments on the relation of stem length to GA effect with green peas.

Alaska peas were grown in vermiculite in a controlled-condition room maintained at 17° C, with a 16 hour photoperiod (8 A.M. to 12 midnight), the light of about 1500 ft-c coming from a bank of mixed fluorescent and incandescent lights. They were automatically subirrigated twice daily with a nutrient solution composed of 120 g "Hyponex" mixture (Hydroponics Chemical Company, Copley, Ohio) per 100 L of tap water. At the age of 14 days, the plants were harvested and trimmed to leafless stems of varying lengths, terminating in an apical bud still enclosed within the stipules (for further details, see Galston and Baker, (4)). These stems were then immersed basally for 1 hour in 10 ml of 4 % sucrose and buffer and 10^{-4} M GA contained in a 50 ml beaker. At the end of this time, apical 5 mm sections were cut with a guillotine, and placed as usual in either 4 % sucrose and buffer, or in sucrose-buffer augmented by 10^{-4} M IAA. The results are shown in figure 8. From these data, it can be seen that growth of the sections is independent of stem length when they are supplied with auxin, but that growth is greater with increasing stem length in the cultures devoid of auxin. Thus, green peas differ in at least 2 ways from etiolated peas: 1) IAA-GA synergism may be observed in very short stem sections, and 2) the interaction of GA with stem tissue, resulting in enhanced growth, is independent of auxin supply. These results, taken together with those of Brian and Hemming (2) showing strict dependence of GA effects on auxin, imply some effect of acropetally-migrating GA on auxin content or activity of the apical green stem tissue.

Discussion

There have recently been several reports of the synergistic interaction of auxins and gibberellins in various biological systems (2, 8, 11, 15, 17, 18), and the evidence now seems strong that GA can generally function only if some auxin or apically-produced growth factor is present. From the present paper it appears that in the etiolated pea epicotyl, this synergism depends on the prior interaction of GA with some other component of the tissue present in relatively short supply throughout the epicotyl. Such a "three factor" interpretation of growth regulation in the pea internode coincides very well with and supports a similar hypothesis recently advanced by Brian and Hemming (2). This hypothetical "third factor"

would seem to be more abundant in green pea stem tissue than in etiolated tissue, for GA-IAA synergism can be demonstrated in very short (5.0 mm) sections of green stem, while much longer pieces of etiolated epicotyl are needed to show the same effect. The fact that, in the absence of added auxin, the effect of GA on green tissue is similarly dependent on length of stem traversed, suggests that the third factor has something to do with auxin metabolism, possibly a "sparing" of auxin.

A sparing action of GA on auxin is suggested by the work of Galston (4) Pilet (13) and Stutz and Watanabe (16), all of whom found GA application to depress the activity of IAA oxidase in various tissues, by the work of Nitsch (12) who found GA to increase the level of auxin in various woody shoots and by the indirect evidence of Vlitos and Meudt (17, 18) with slightly decapitated peas. Galston (4) was able to show that the depression of IAA oxidase activity was due to enhanced levels of a substance, presumptively phenolic, which acts as an inhibitor of auxin destruction. Gortner and colleagues (7) have recently reported that ferulic acid (3-methoxy-4-hydroxy-cinnamic acid), a phenolic substance, functions in this manner in the pineapple. The indication that this "third factor" is more abundant in green than in etiolated pea tissue also parallels the situation already reported for the inhibitor of IAA oxidase (4). The quantity of IAA oxidase inhibitor in etiolated peas is also known to be controlled by the red-far-red reversible photoreaction (9).

It should also be noted that certain growth effects of GA cited in the literature, such as promotion of germination of lettuce seeds, expansion of excised bean leaf discs and some responses of excised stem sections appear not to be easily explicable in terms of an interaction of GA with auxin metabolism. Brian and Hemming (2) have also adduced considerable evidence against auxin-sparing as a mode of action of GA, based on the fact that GA evokes responses which cannot be produced by any concentration of externally applied IAA. Their arguments, while cogent, are not completely convincing because of the difficulty of equating auxin concentration and localization in the cell with that of synthetic auxin applied to the exterior surface of a mass of tissue. In any event, we do not wish to advocate auxin-sparing as a universal mode of action of GA, but would like to indicate that, in the pea plant, there is good evidence that GA causes cells to produce a substance which acts, in vitro, like an auxin-sparing substance, and acts in vivo like a promoter of growth and of GA-IAA synergism.

Although it is tempting to ascribe all of the effects reported in the present paper to actual movement of GA up the epicotyl, such a transport has not been unequivocally demonstrated in these experiments. The possibility must still be entertained that basally-applied GA does not actually reach the apex, but rather sets in motion a chain of chemical events which move rapidly up the epicotyl to the region excised and used in the growth studies. We intend to investigate this situation by the use of tritium-labelled gibberellic acid.

Summary

1. When GA and IAA are applied jointly to excised 5 mm-long epicotyl sections of etiolated peas, the growth effects produced are no greater than additive, and are frequently less than additive. If, however, GA is applied basally to an excised 100 mm-long epicotyl, and the IAA subsequently applied to the excised apical section, the combined growth effects are much more than additive (synergistic).

2. The epicotyls react to as little as 10^{-7} M GA, half maximally to 10^{-6} M GA and optimally to 10^{-5} and 10^{-4} M GA. They can thus be used to assay GA in this range. The volume of solution to be assayed per epicotyl need not exceed .5 ml, and can thus be used to detect down to .03 μg of GA.

3. Appreciable effects of basally-applied GA are evident at the apex, 100 mm away, within 3 to 5 minutes after application, indicating a transport rate as rapid as 20 to 33 mm/min.

4. The effects of GA in such an etiolated epicotyl is directly proportional to the length of epicotyl between the base, which received the GA, and the apex, which received the IAA. In green stems, where GA-IAA synergism is clearly shown in excised 5 mm sections, GA effects are dependent on the length of stem traversed, even if no auxin is supplied to the excised section.

5. These results can be interpreted in terms of a "third factor" required for GA-IAA interaction. Such a factor, apparently limiting in etiolated pea stem tissue and abundant in green tissue, may operate through an auxin-sparing mechanism.

Literature Cited

1. Brian, P. W. and Hemming, H. G. A relation between the effects of gibberellic acid and indolylacetic acid on plant cell extension. Nature 179: 417. 1957.
2. Brian, P. W. and Hemming, H. G. Complementary action of gibberellic acid and auxins in pea internode extension. Ann. Bot. (*N.S.*) *22*: 1–17. 1958.
3. Brian, P. W., Hemming, H. G. and Radley, M. A physiological comparison of gibberellic acid with some auxins. Physiol. Plantarum 8: 899–912. 1955.
4. Galston, A. W. Investigations on a substance whose level in tissue is controlled by the red-far-red reversible photoreaction. In: Photoperiodism and Related Phenomena in Plants and Animals. An international symposium held at Gatlinburg, Tenn., Oct. 1957. (In press, 1958.)
5. Galston, A. W. and Baker, R. S. Studies on the physiology of light action. IV. Light enhancement of auxin-induced growth in green peas. Plant Physiol. *26*: 311–317. 1951.
6. Galston, A. W. and Hand, M. E. Studies on the physiology of light action. I. Auxin and the light inhibition of growth. Amer. Jour. Bot. 36: 85–94. 1949.
7. Gortner, W. A., Kent, M. J. and Sutherland, G. K. Ferulic and *p*-coumaric acids in pineapple tissue as modifiers of pineapple indoleacetic acid oxidase. Nature 181: 630–631. 1958.

8. HAYASHI, T. and MURAKAMI, Y. Studies on the physiological action of gibberellins (Part I). Bull. Natl. Inst. Agr. Sci. D (Japan): 159–197. 1958.
9. HILLMAN, W. A. and GALSTON, A. W. Inductive control of indoleacetic acid oxidase activity by red and near infrared light. Plant Physiol. 32: 129–135. 1957.
10. KATO, J. Studies on the physiological effect of gibberellin. I. On the differential activity between gibberellin and auxin. Mem. Coll. Sci. Univ. Kyoto Ser. B. 20: 189-193. 1955.
11. LUCKWILL, L. C. Fruit growth in relation to internal and external chemical stimuli. Paper presented at Mt. Holyoke College, June 1958. Seventeenth Growth Symposium (In press.)
12. NITSCH, J. P. Growth responses of woody plants to photoperiodic stimuli. Proc. Amer. Soc. Hort. Sci. 70: 512–525. 1957.
13. PILET, P.-E. Action des gibberellines sur l'activité auxin-oxydasique de tissus cultivé in vitro. Compt. rend. acad. sci., (France) 245: 1327–1328. 1957.
14. PURVES, W. K. and HILLMAN, W. S. Response of pea stem sections to indoleacetic acid, gibberellic acid and sucrose as affected by length and distance from apex. Physiol. Plantarum 11: 29–35. 1958.
15. SCHROEDER, C. A. and SPECTOR, C. Effect of gibberellic acid and indoleacetic acid on growth of excised fruit tissue. Science 126: 701–702. 1957.
16. STUTZ, R. E. and WATANABE, R. The gibberellins II. The effect of gibberellic acid and photoperiod on indoleacetic acid oxidase in *Lupinus albus* L. Argonne Natl. Lab., Biol., and Med. Research Div., Semiannual Report Jan.–June 1957, 107–109.
17. VLITOS, A. J. and MEUDT, W. Relationship between shoot apex and effect of gibberellic acid on elongation of pea stems. Nature 180: 284. 1957.
18. VLITOS, A. J. and MEUDT, W. The effect of light and of the shoot apex on the action of gibberellic acid. Contrib. Boyce Thompson Inst. 19: 55–62. 1957.

FOLIAR UPTAKE OF SALT CONSTITUENTS OF WATER BY CITRUS PLANTS DURING INTERMITTENT SPRINKLING AND IMMERSION [1, 2]

FRANK M. EATON AND R. B. HARDING

DEPARTMENT OF SOILS AND PLANT NUTRITION, UNIVERSITY OF CALIFORNIA CITRUS EXPERIMENT STATION, RIVERSIDE, CALIFORNIA

On the basis of diverse methods of application and appraisal, the now rather extensive literature on foliar uptake gives one the impression that almost any substance in soluble or gaseous form may be absorbed by plant leaves. In some measure, the materials are translocated from the point of application to surrounding or other tissues. Boynton's review (1) places emphasis on the value and limitations of foliar application of plant nutrients. Bukovac and Wittwer (3), with a short review of pertinent literature, present new data on the uptake of 14 elements studied by means of radioactive isotopes. Van Overbeek (9) has reviewed papers dealing with the foliar absorption and translocation of plant regulators. He introduces his review with a critical analysis of mechanisms of solute entry into leaves in the light of cuticular compositions and structures. The foliar uptake of herbicides, fungicides, sulfur dioxide, fluoride, and smog constituents have all received recurrent attention.

The present paper has its background in two somewhat distinct fields of interest: 1) the accumulation of the salt constituents of natural waters by citrus leaves under sprinkler irrigation, and 2) the selective absorption and constancy of foliar cation accumulations.

As regards sprinkler irrigation, Harding et al (4) have shown that under some conditions there is an excessive accumulation of sodium and chloride in citrus leaves when orchards are irrigated with sprinklers. The sprinklers customarily used are the type which make a full circle every 2 to 4 minutes. Between successive wettings there is opportunity for evaporation from the film left on the leaves. This latter fact gave rise to a question of whether the salts in the original irrigation waters were sufficiently concentrated to have caused the observed accumulations. Evaporation from the film remaining on leaves between successive wettings would result in high salt concentrations on the leaf surfaces.

Comparisons between day versus night sprinkling and intermittent versus continuous sprinkling are reported in this paper. Absorption was measured when the tops of small, rooted lemon cuttings and orange seedlings in potting soil were inverted for successive overnight periods in various single-salt solutions. The foregoing studies gave opportunity for appraising the constancy of cation absorption by leaves for comparison with absorption through roots as studied by others. This latter subject, as recently developed by Kretschmer et al (5), led to their statement that "increase in the absorption of any one cation results in reduction in absorption of some other cation or cations or increase in uptake of one or more anions."

MATERIALS AND METHODS

Budded Valencia orange trees growing in 5-gallon cans of soil were used for the sprinkling experiments. Rooted Eureka lemon cuttings and seedling sweet orange trees, both 6 to 8 inches tall and growing in 1-quart containers of potting soil were inverted over 3-liter jars of aerated solution and tops immersed for 6 periods of 18 hours each, between 3:00 P.M. and 9:00 A.M.

[1] Received June 27, 1958.
[2] Paper no. 1050, University of California Citrus Experiment Station, Riverside, California.

TABLE I

FOLIAR ABSORPTION BY YOUNG VALENCIA ORANGE TREES INTERMITTENTLY SPRINKLED WITH SYNTHETIC IRRIGATION WATER* FOR NINE 6-HOUR PERIODS DURING THE DAY AND DURING THE NIGHT (OCTOBER, 1957)

SPRINKLING TREATMENT	LEAF CONSTITUENTS, MEQ/100 G DRY WEIGHT						
	CATIONS					ANIONS	
	Na	K	Mg	Ca	TOTAL	Cl	TOTAL S AS SO_4
			Young leaves				
Control	3.0	49.4	38.5	155	246	2.3	8.8
Daytime	8.7	40.0	41.0	195	285	8.3	13.5
Nighttime	4.8	42.2	39.4	188	274	3.8	13.4
			Mature Leaves				
Control	5.2	43.5	36.9	220	306	2.3	11.2
Daytime	13.2	40.1	38.5	230	322	9.5	15.3
Nighttime	7.6	43.8	36.9	220	308	4.9	22.2
			Meq/l				
* Irrigation water (plus HCO_3 -- 2.5)	7.1	0.2	3.4	4.3	15.0	3.8	8.0

All leaves in a given sample were individually washed and rinsed with distilled water. This procedure removed any precipitated or soluble salts present on the exterior surface of the leaves. This method of leaf washing has been employed in this laboratory for 20 years and has been subjected to thorough testing (10). The ash of 1 g of dried leaf material was taken up in 100 ml of dilute HCl by standard procedures, and Na, K, and Ca were determined by means of a Perkin-Elmer flame photometer, using Li as an internal standard. Magnesium was determined colorimetrically (11), Cl was determined by titration with $AgNO_3$ (2), and total S was determined as $BaSO_4$ (8). Water solutions were analyzed by methods described in USDA Handbook 60 (8) for analysis of irrigation waters.

RESULTS AND DISCUSSION

Citrus leaves are glabrous. When first wet by spraying or dipping, there remains on the leaves a mixture of film and droplets. With continued spraying the films become persistent over much of the leaf surface. Turrell (7) has pointed to the improbability of rain water entering the intercellular spaces of citrus leaves, both because of the high angle that the guard cells make with intervening leaf surface and because of the resinous plugs which develop in the

TABLE II

FOLIAR ABSORPTION BY VALENCIA ORANGE TREES SPRINKLED WITH SYNTHETIC IRRIGATION WATER* 4 HOURS PER DAY, CONTINUOUSLY (TOTAL, 16 HOURS) AND INTERMITTENTLY (TOTAL, 4 HOURS) IN LATE SUMMER

SPRINKLING TREATMENT	LEAF CONSTITUENTS, MEQ/100 G DRY WEIGHT						
	CATIONS					ANIONS	
	Na	K	Mg	Ca	TOTAL	Cl	TOTAL S AS SO_4
			*Developing leaves (current cycle)***				
Continuous	2.6	45.3	36.1	110	194	1.7	17.6
Intermittent (1 sec/30 sec)	23.4	37.1	33.6	170	264	16.8	15.0
			Young leaves (previous cycle)				
None (control)	6.1	44.0	24.6	240	315	0.6	9.4
Continuous	8.2	30.7	45.1	215	299	1.4	11.8
Intermittent (1 sec/30 sec)	28.8	30.0	41.8	190	291	25.2	17.2
			Meq/l				
* Irrigation water (plus HCO_3 — 2.5)	7.1	0.2	3.4	4.3	15.0	3.8	8.0

** These leaves were too small at the start of the experiment for good control samples.

stomatal openings. Only occasionally during plant immersion was water found to have entered the intercellular spaces of the leaves. Such leaves were not included in the samples for analysis.

When an orange twig with a number of leaves is sprinkled or dipped in water and then shaken lightly, there is a gain of 15 to 20 % in fresh weight. When leaves covered with these water films are exposed to the sun and wind for a few minutes, much of the water is evaporated.

The composition of the sprinkler water used in the experiments reported in tables I and II is similar to that of some of the waters which have caused trouble in orchards (4).

Under intermittent sprinkling (table I), all leaves showed greater accumulation of Na^+ and of Cl^- during the day than during the night. There was some absorption of Ca^{++} and of SO_4^{--} and a loss of K^+ by the young leaves. In the mature leaves there were only small differences in the amounts of Ca^{++}, but increases in the amounts of SO_4^{--}. Only small differences in the amounts of K^+ are apparent in the mature leaves. The variability in loss of K in the different experiments is in keeping with previous observations by Miller (6). This experiment was conducted in late October, when the temperatures were moderate, humidity was fairly high, and wind velocities at night were usually low. There was never more than a partial drying of the leaves between successive sprayings.

The data of table II are from an experiment conducted in late summer, when there was a great deal of evaporation between the successive applications of water. When the sprinkling was intermittent, the developing leaves absorbed Na^+ and Cl^- heavily. The leaves of the previous cycle (young leaves) also ac-

TABLE III

FOLIAR ABSORPTION BY ROOTED EUREKA LEMON CUTTINGS WHEN INVERTED WITH TOPS IMMERSED IN AERATED SALT SOLUTIONS FOR SIX 18-HOUR OVERNIGHT PERIODS

SOLUTION SALT	CONC (MEQ/L)	LEAF CONSTITUENTS, MEQ/100 G DRY WEIGHT					
		CATIONS					ANION
		Na	K	Mg	Ca	TOTAL	Cl
		New leaves					
None (air)	...	4.7	38.4	24.6	140	208	3.1
Tap water*		4.3	38.4	27.9	135	206	3.1
NaCl	50	18.2	38.4	25.4	125	207	15.2
"	100	26.7	41.0	23.0	115	206	24.2
KCl	50	4.7	107.5	23.0	115	250	69.6
$MgCl_2$	50	4.7	34.6	32.8	140	212	16.1
$CaCl_2$	50	4.7	45.3	23.0	120	193	24.2
		Old leaves					
None (air)	...	4.3	20.4	24.6	275	324	2.8
Tap water*		4.3	16.6	27.1	285	333	1.7
NaCl	50	9.8	14.6	29.5	320	374	6.8
"	100	18.9	14.6	24.6	320	378	16.6
KCl	50	4.7	49.9	24.6	285	364	36.9
$MgCl_2$	50	3.9	10.2	44.3	340	398	13.0
$CaCl_2$	50	4.3	17.9	36.1	320	378	11.6
		Stems					
None (air)	...	5.6	14.6	15.6	110	146	1.4
Tap water*		6.1	14.6	14.8	105	141	1.1
NaCl	50	6.5	14.6	18.0	105	144	3.1
"	100	6.5	10.2	13.1	110	140	2.8
KCl	50	5.6	23.0	15.6	105	149	17.2
$MgCl_2$	50	4.8	14.6	16.4	105	141	3.1
$CaCl_2$	50	5.6	10.2	14.8	105	136	4.2
		Roots					
None (air)	...	8.7	24.3	41.2	65	139	13.2
Tap water*		8.2	21.8	45.1	65	140	11.0
NaCl	50	7.4	16.6	46.7	65	136	11.6
"	100	6.1	21.8	35.3	65	128	8.7
KCl	50	9.5	25.6	36.9	65	137	11.0
$MgCl_2$	50	8.6	20.4	36.9	65	131	8.7
$CaCl_2$	50	10.8	32.0	44.3	65	152	11.0
		Meq/l					
* Tap water (plus HCO_3 — 2.50 and SO_4 — 0.54)		2.20	0.08	0.25	1.36	3.89	0.55

cumulated much Na^+ and Cl^-, and there was a loss of K^+. With continuous sprinkling, on the other hand, the accumulations of Na^+ and Cl^- were minor. The intermittently sprayed leaves became coated with precipitated salts of low solubility.

The foregoing experiments establish beyond reasonable question that the concentrations of ions in the water supply were too low to cause an appreciable uptake of ions; only when the concentrations were increased in the films by evaporation were there extensive absorptions. In the interest of attaining more tangible evidence on concentration relations for foliar uptake, young citrus plants were inverted and their tops immersed in aerated solutions of known concentrations. The tops of the plants were exposed to the air for 6 hours during the middle of each day and they maintained a healthy appearance.

The lemon cuttings of table III and the orange seedlings of table IV showed a similarity of pattern in foliar uptake of Na^+ and Cl^-, but the lemons took up less sodium than the oranges. When the tops of these plants were in 50 meq/l solutions of KCl, $MgCl_2$ and $CaCl_2$, there were both likenesses and differences in the behavior of the two species. Both species responded with rather large accumulations of K^+ in the KCl solution. The Mg^{++} accumulations in the $MgCl_2$ solution were moderate. The orange leaves were outstanding in their Ca^{++} absorptions; they gained in calcium to the extent of 100 meq per 100 g dry weight, and with this there was a 50 meq increase in Cl^-. The lemons accumulated little or no Ca^{++}. Stems (also immersed) of both species tended to follow on a lesser scale the absorptions which occurred in the leaves. The only well-defined example of translocation from the stems to the roots is represented by the gains in Na^+, K^+, and Cl^- in the instance of the sweet orange; the lemons did not show this.

The sum of cations tended to remain rather uniform except in the heavy uptake of KCl by the new lemon leaves and of $CaCl_2$ by the orange leaves. Usually there was a decrease in Ca when either Na or K was absorbed in large amounts in leaves. Where Mg was absorbed there was little or no reduction in any other cation in leaves.

The data support well, and carry over to foliar absorption, the modification in the constancy of cation accumulations suggested by Kretschmer et al (5) to cover those instances where a heavy uptake occurs in some anion. They suggested substituting for the sum-of-cations concept the ratio of sum-of-cations to sum-of-anions.

The paper by Harding et al (4) on sprinkler injury reported greater injury in drier inland areas than along the coast, greater injury in the exposed outside

TABLE IV

FOLIAR ABSORPTION BY SWEET ORANGE SEEDLINGS WHEN INVERTED WITH TOPS IMMERSED IN AERATED SOLUTIONS FOR SIX 18-HOUR OVERNIGHT PERIODS

SOLUTION SALT	CONC (MEQ/L)	LEAF CONSTITUENTS, MEQ/100 G DRY WEIGHT					
		CATIONS					ANION
		Na	K	Mg	Ca	TOTAL	Cl
		All leaves					
Tap water*		7.4	50.4	25.4	165	248	1.7
NaCl	50	15.2	53.8	20.5	160	250	9.0
"	100	33.8	53.2	19.7	145	252	21.4
KCl	50	6.1	87.0	19.7	130	243	23.4
$MgCl_2$	50	5.6	49.4	33.6	175	265	17.2
$CaCl_2$	50	6.1	49.2	23.8	275	354	73.6
		Stems					
Tap water*		4.7	39.4	23.0	120	187	3.1
NaCl	50	7.7	38.9	20.5	105	172	7.3
"	100	13.7	42.0	21.3	105	182	14.9
KCl	50	6.9	52.2	18.9	105	183	17.8
$MgCl_2$	50	4.3	37.6	28.7	110	181	12.1
$CaCl_2$	50	5.6	39.4	22.1	130	197	29.3
		Roots					
Tap water*		7.7	27.1	36.1	20	91	7.6
NaCl	50	9.5	23.0	41.8	25	99	9.9
"	100	12.5	26.6	26.2	25	90	11.8
KCl	50	9.0	38.9	31.2	25	104	10.7
$MgCl_2$	50	6.6	32.0	31.2	25	95	15.2
$CaCl_2$	50	8.1	28.7	29.5	25	91	15.2
		Meq/l					
* Tap water (plus HCO_3 — 2.5 and SO_4 — 0.54)		2.20	0.08	0.25	1.36	4.80	0.55

rows of orchards than within the orchards, and the formation of precipitates of only slightly soluble salts on the surfaces of leaves in exposed positions. In the present studies this precipitation occurred on the leaves of the oranges sprayed for 1 second every 30 seconds, but there was none on the leaves under continuous irrigation. Continuous sprinkling is known to be practiced without injury in situations where the same water supply intermittently applied causes severe injury.

Summary

Lemon and orange leaves accumulated more sodium and chloride when irrigated with sprinklers of the intermittent type during the day than they did when similarly irrigated during the night. They accumulated more when intermittently sprinkled than when continuously sprinkled. Intermittent sprinkling permitted evaporation and concentration of the salts in the water films left on the leaves. In advance of the measurements it was thought that little accumulation could be expected from the concentrations of salts in the initial irrigation water, and this proved to be the case.

Comparisons were made of absorption by orange seedlings and by rooted lemon cuttings when the plants were inverted and the tops immersed for overnight periods in aerated single-salt solutions. Foliar absorptions by this procedure supported the earlier results and gave a constancy of cation accumulation except when there were heavy accumulations of chloride in association with particular cations.

The writers are indebted to Dr. A. P. Vanselow for the design of the timer for the intermittent sprinklers and to Mr. G. W. McLean for analyses.

Literature Cited

1. Boynton, D. Nutrition by foliar application. Ann. Rev. Plant Physiol. 5: 31–54. 1954.
2. Brown, J. G. and Jackson, R. K. A note on the potentiometric determination of chloride. Proc. Amer. Soc. Hort. Sci. 65: 187. 1955.
3. Bukovac, M. J. and Wittwer, S. H. Absorption and mobility of foliar applied nutrients. Plant Physiol. 32: 428–435. 1957.
4. Harding, R. B., Miller, M. P. and Fireman, M. Absorption of salts by citrus leaves during sprinkling with water suitable for surface irrigation. Proc. Amer. Soc. Hort. Sci. 71: 248–256. 1958.
5. Kretschmer, S. J., Toth, S. J. and Bear, F. E. Effect of chloride versus sulfate ions on nutrient-ion absorption by plants. Soil Sci. 76: 193–199. 1953.
6. Miller, E. C. A physiological study of the winter wheat plant at different stages of its development. Kansas State College Tech. Bul. 47. 167 pp. 1939.
7. Turrell, F. M. Citrus leaf stomata: structure, composition, and pore size in relation to penetration of liquids. Bot. Gaz. 108: 476–483. 1947.
8. U.S. Salinity Laboratory Staff Diagnosis and improvement of saline and alkali soils, L. A. Richards, ed. U.S.D.A. Handbook 60. Govt. Printing Office, Washington 25, D. C. 1954.
9. Van Overbeek, J. Absorption and translocation of plant regulators. Ann. Rev. Plant Physiol. 7: 355–372. 1956.
10. Wallihan, E. F. Iron chlorosis, what is it? Citrus Leaves 33: 12–13. 1953.
11. Young, H. Y. and Gill, R. F. Determination of magnesium in plant tissue with thiazole yellow. Anal. Chem. 23: 751–754. 1951.

METABOLIC STUDIES OF APPLIED INDOLEACETIC ACID-1-C^{14} IN PLANT TISSUES AS AFFECTED BY LIGHT AND 2,4-D TREATMENT[1, 2, 3]

S. C. FANG, PATRICIA THEISEN and JOSEPH S. BUTTS

Department of Agricultural Chemistry, Oregon Agricultural Experiment Station, Oregon State College, Corvallis, Oregon

Phototropic response of plants or plant organs to unilateral light is generally explained by the unequal distribution of growth hormones. The mechanisms which will bring about such asymmetric concentration may be from any one or a combination of the following changes; namely, the lateral redistribution, the synthesis, and the destruction of auxins in response to light (12). The formation of auxins in response to light and their disappearance in darkness would produce a negative curvature; therefore, this mechanism appears to be a less significant part of the phototropic reaction. Lateral redistribution of auxins in response to light has been demonstrated by several workers (3, 13). Recently, this problem was reinvestigated using radioactive indoleacetic acid (4, 10). The results did not support the concept of lateral transport. It was also found by Ching and Fang (5) that regardless of the concentration of absorbed radioactive indoleacetic acid, stimulation time, and plant species,

[1] Received July 7, 1958.
[2] This work was supported by contract no. AT (45-1)-304 United States Atomic Energy Commission.
[3] Technical Paper no. 1155, Oregon Agricultural Experiment Station.

no appreciable unequal distribution of radioactivity in geotropically stimulated organs was observed. Therefore, the light-induced destruction of auxins may play a more important role in phototropism than has been indicated (12).

It has been reported that Avena coleoptiles did not respond to either phototropic or geotropic stimulation if they were treated with 2,4-D (11). No satisfactory explanation was given. If photo-inactivation of auxins plays a primary role in phototropism, it would seem that 2,4-D treatment must have affected or destroyed the mechanism causing increased destruction of auxin by light. Therefore, there was no unequal distribution of auxin between the light and dark sides of the plant organ.

It is now well known that 3-indoleacetic acid is a naturally occurring auxin in the plant world. It seems that an investigation in detail of the metabolic fate of the IAA which is administered to tissues may result in a better understanding of its mode of action. The present experiments were undertaken 1) to determine the effect of light on the destruction of indoleacetic acid by excised plant shoots and roots, 2) to determine the effect of 2,4-D treatment on the light induced destruction of IAA, and 3) to analyze in detail the metabolic fate of the absorbed indoleacetic acid-1-C^{14} under various conditions. If the destruction of exogenous auxin by plant organs should be influenced by light, then the destruction of endogenous auxin probably would be influenced similarly.

Materials and Methods

Dry pea seeds (*Pisum sativum* L. var. Alaska) and corn seeds (*Zea mays* L. var. Golden Cross) were soaked in distilled water for 2 hours and were then germinated in the dark between 2 sheets of moist paper. The paper was rolled and set in an upright position in the incubation chamber, so that the growths of root and shoot would not be affected by geotropic forces. Uniform seedlings were removed from the paper after 3 or 4 days and immersed in beakers containing either 0.01 M phosphate buffer (pH 5.2), or buffer plus 10^{-4} M 2,4-D, with aeration. For treatment of longer than 5 hours, the following method was used. Uniform seedlings were rerolled between 2 sheets of paper which were moistened either with buffer or with buffer plus 2,4-D. At the end of the treatment, the seedlings were removed and washed in distilled water. The apical 1.0 to 1.5 cm of shoot tips was cut off, blotted dry on filter paper and weighed. In the experiments with root tissue the distal 2 cm of the roots was used. Four equal samples of shoot tips or root tips (2 samples each from buffer-treated and buffer plus 2,4-D-treated seedlings) were placed in 50 ml Erlenmeyer flasks which contained 10 ml of 0.01 M phosphate buffer, pH 5.2 and 234 μg IAA-1-C^{14}. Immediately after adding the sample the flask was connected to a CO_2 scraper, so that the respiratory CO_2 from the flask was continuously swept out by a stream of CO_2-free air at the rate of 20 to 25 ml per minute and absorbed in NaOH solution, which was changed periodically. The flasks were shaken continuously at room temperature, 24 to 26° C. The respiratory CO_2 was precipitated as $BaCO_3$ and the radioactivity determined in the usual manner. At the end of the incubation period, the tissues were removed from the ambient solutions, rinsed with water and homogenized with ethyl alcohol. The radioactivities of the ambient solution, alcohol extract of the tissues, and the residue of the tissue were also determined by the conventional method. In the 2nd experiment with corn shoots, the apical 10 mm tips were cut into equal halves and were used in the dark and the light experiments respectively. All manipulations were carried out under dim light at room temperature. A 20-watt fluorescent light placed 8 inches from the flask was used for illumination during the incubation period.

The alcohol extracts and the ambient solutions after incubation were chromatographed on Whatman no. 1 filter paper in an isopropyl alcohol, concentrated ammonium hydroxide, water solvent (10 : 1 : 1 v/v). Ascending chromatography was used throughout. Radioautographs were prepared in the usual way using Eastman No-screen x-ray film. The film was exposed to the chromatograms for periods varying from 2 weeks to 2 months depending on the radioactivity. The distribution of radioactive spots was determined either by direct scanning of the paper chromatograms or by measuring the density of the spots from the radioautographs. A regular radiochromatogram scanner and a Photovolt densitometer were used for these purposes. The IAA concentration in the tissues at the end of the runs was then calculated from the percentage of radioactivity in the form of IAA on the chromatogram, the radioactivity of the ethanol extract, and the specific activity of the administered IAA.

Results and Discussion

The rate of IAA destruction by normal corn and pea shoots as measured from the radioactivities of respiratory CO_2 was significantly increased by light. In corn shoots the destruction of IAA during the 1st 6-hour period was increased 51 % in both runs by exposure of the reaction to light, whereas in pea shoots the destruction was increased 48 % by light in the 1st run, and 23 % in the 2nd run. There was a great variation between the 2 runs in the amount of IAA destroyed per unit weight of pea shoots. This variation might be due to the age of the seedlings and the length of the tips used. Four of the 6 experiments were carried on for 19 hours, at which time the radioactivities in the ambient solutions were almost exhausted; at the end of 19 hours the over-all effect of light on the destruction of IAA as measured by respiratory $C^{14}O_2$ was not as great as the 6-hour measurement. Results also indicated that 2,4-D treatment prevented a light-induced increase in IAA destruction in both corn and pea shoots. This may be the chief reason for a lack of phototropic response to lateral light by some 2,4-D treated plant organs. Table I presents the rate of oxidative decarboxylation

of IAA-1-C^{14} by corn and pea shoots as expressed by radioactivity of $C^{14}O_2$ evolved per time interval, and the ratio of oxidative decarboxylation in light to oxidative decarboxylation in dark per time interval. The influence of light on this mechanism in buffer-treated tissues appears to be more pronounced during the 1st 3 hours of incubation than at the 6-hour period, as indicated by the L/D ratio. A comparison between the buffer-treated and buffer plus 2,4-D-treated shoot tips reveals that in the flasks exposed to light the radioactivity of the respiratory CO_2 from 2,4-D-treated tissues was generally less than that from the control tissue. However, the trend was completely reversed when the reaction was conducted in complete darkness. The effect of 2,4-D on the destruction of exogenous IAA has been investigated in vivo in bean and corn plants (6). Treatment with 2,4-D decreased the rate of oxidative decarboxylation of IAA in these 2 species. It has been suggested that the action of 2,4-D on IAA destruction is probably due to an indirect effect, which inhibited the synthesis of IAA oxidase or other cofactors involved in the destruction of IAA. The results obtained from this experiment fail to support fully this view because the 2,4-D-treated shoots destroyed more IAA than the control shoots when the incubations were carried out completely in darkness. In an investigation on the nature of the effect of light on the enzymatic reaction, Galston and Baker (8) have found that there is a natural inhibitor to the reaction in pea tissues, and that light serves to overcome the effect of this inhibitor. It is possible that 2,4-D treatment might also overcome the effect of this inhibitor which would explain why light had no effect on the enzymatic reaction in 2,4-D treated shoots.

Table II presents the rate of oxidative decarboxylation of IAA by the root tips of corn and pea seedlings. The L/D ratios of the oxidative decarboxylation of IAA during the 1st 6-hours of incubation were near unity with either corn roots or pea roots. Apparently light does not participate in this reaction. Slight reductions in IAA destruction were noted

TABLE I

INFLUENCE OF LIGHT ON THE OXIDATIVE DECARBOXYLATION OF INDOLEACETIC ACID-1-C^{14} BY NORMAL AND 2,4-D-TREATED CORN AND PEA SHOOTS

INCUBATION TIME HOURS	CORN SHOOT, CPM × 10^3						PEA SHOOT, CPM × 10^3					
	1ST RUN			2ND RUN			1ST RUN			2ND RUN		
	LIGHT	DARK	L/D	LIGHT	DARK	L/D	LIGHT	DARK	L/D	LIGHT	DARK	L/D
Control												
1	2.55	0.62	4.10	2.38	1.68	1.42	7.61	4.82	1.58	12.03	8.83	1.36
2	3.65	1.47	2.48	1.55	0.75	2.07	14.08	8.33	1.69	14.66	10.04	1.46
3	2.17	1.50	1.45	3.06	1.96	1.56	14.76	9.45	1.56	17.29	14.51	1.19
6	10.42	8.86	1.18	5.10	3.63	1.40	42.47	30.99	1.37	54.53	46.00	1.18
Total	18.79	12.45	1.51	12.09	8.02	1.51	78.92	53.59	1.48	98.51	79.38	1.23
2,4-D treated												
1	1.74	1.48	1.18	2.00	1.34	1.49	5.69	6.63	0.86	6.84	7.76	0.88
2	3.99	2.82	1.41	1.43	1.05	1.36	11.58	12.57	0.92	13.51	13.07	1.03
3	1.80	2.43	0.74	3.04	2.81	1.08	13.42	14.94	0.92	18.17	15.83	1.15
6	9.38	10.72	0.87	4.54	7.75	0.59	26.88	32.98	0.82	52.52	44.66	1.17
Total	16.91	17.45	0.97	11.01	12.95	0.85	57.57	67.12	0.86	91.04	81.32	1.12

TABLE II

INFLUENCE OF LIGHT ON THE OXIDATIVE DECARBOXYLATION OF INDOLEACETIC ACID-1-C^{14} BY NORMAL AND 2,4-D-TREATED ROOT TISSUES

INCUBATION TIME HOURS	CORN ROOT, CPM × 10^3			PEA ROOT, CPM × 10^3		
	LIGHT	DARK	L/D	LIGHT	DARK	L/D
Control						
1	21.40	19.00	1.13	60.89	58.92	1.03
2	24.05	26.69	0.90	31.07	28.78	1.08
3	15.94	17.55	0.91	15.05	14.39	1.05
6	20.36	21.50	0.95	5.64	5.15	1.10
Total	81.75	84.74	0.96	112.65	107.24	1.05
2,4-D treated						
1	19.13	17.08	1.12	57.95	58.97	0.98
2	27.51	21.20	1.30	27.54	25.60	1.08
3	15.39	15.46	1.00	6.03	7.02	0.86
6	22.12	19.79	1.12	1.43	1.49	0.96
Total	84.15	73.53	1.14	92.95	93.08	1.00

both in pea and in corn roots from 2,4-D-treated seedlings. With equal weight of root tips, the destruction of IAA by pea roots was considerably higher than the destruction by corn roots, possibly indicating that the auxin-destroying enzyme is more abundant in pea roots.

Table III presents the distribution on a percentage basis of radioactivity in the respiratory CO_2, the tissue extract, the insoluble residue of tissues and the medium after incubation. The radioactivity from respiratory CO_2 represents the oxidative decarboxylation of IAA, whereas the radioactivity in the tissue extract may be derived from free IAA, bound IAA and other metabolites. It is logical to assume that the absorption of IAA from the medium by floating tissues precedes its oxidation. The disappearance of radioactivity from the medium after incubation should represent the amount of IAA taken up by the tissues. The ratio (O/A) between the oxidation and the absorption of IAA should provide a relative index for expressing the enzymic activity of IAA oxidase in excised tissues. It is surprising to note that the O/A values for pea shoots are rather constant between 2 runs and therefore are not dependent on the incubation time. Cutting the tip in halves definitely increased the amount of absorption by corn shoot tissues and perhaps, also, decreased the rate of decarboxylation. Treatment with 10^{-4} M 2,4-D did not change the O/A ratio significantly. With buffer-treated shoots the O/A ratios obtained from light runs were always higher than those from dark runs. This would suggest that light may influence only the oxidation of IAA but not the absorption. Again, no appreciable differences in O/A ratios for corn and pea roots were found between the light and the dark experiments which indicates that a completely different mechanism may be operating in the root tissue.

When a comparison is made between the 2 species, it is interesting to note that the O/A ratio of pea tissues is considerably higher than that of corn tissues. A difference in the metabolic pathway of IAA breakdown in plant cells might be responsible for this variation. Oxidative degradation of IAA is apparently the main avenue of IAA breakdown in pea tissues, whereas in corn the formation of indoleacetylaspartic acid and other metabolites is predominant. As shown

TABLE III

INFLUENCE OF LIGHT ON THE DISTRIBUTION OF RADIOACTIVE CARBON FROM IAA-1-C^{14} BY NORMAL AND 2,4-D-TREATED PLANT TISSUES

Experiment	Tissues	Wt of tissue used	Duration of incubation	Pre-treatment with 10^{-4} M 2,4-D	Light or dark	O/A	% recovery of C^{14} from IAA-1-C^{14}				
							Respiratory CO_2	Ethanol extract of tissue	Residue of tissue	Ambient solution after incubation	Total recovery
		g	hr	hr							
1	Corn shoot tips	2.11	19	0	L	0.33	30.97	61.79	1.95	9.21	103.92
		2.10	19	0	D	0.27	24.69	65.11	1.83	8.47	100.10
		2.12	19	5	L	0.38	30.50	48.66	2.17	10.79	92.12
		2.00	19	5	D	0.42	36.22	48.37	2.60	11.00	98.19
2	Corn shoot tips	1.52	6	0	L	0.10	6.36	57.73	0.96	32.04	97.09
		1.44	6	0	D	0.08	4.23	51.18	0.88	35.33	91.62
		1.48	6	16	L	0.10	5.79	53.22	0.81	32.57	92.39
		1.58	6	16	D	0.11	6.82	56.38	1.26	29.74	94.20
3	Pea shoot tips	2.31	19	0	L	0.87	77.22	10.36	1.33	18.82	107.73
		2.29	19	0	D	0.79	69.22	16.71	1.63	15.13	102.69
		2.35	19	5	L	0.80	65.79	15.30	1.27	17.63	99.99
		2.31	19	5	D	0.80	66.86	15.72	1.30	13.16	97 04
4	Pea shoot tips	1.30	6	0	L	0.86	51.32	7.76	0.77	42.05	101.90
		1.32	6	0	D	0.83	41.78	7.87	0.55	49.37	99.57
		1.31	6	16	L	0.85	47.91	8.05	0.52	41.53	98.01
		1.37	6	16	D	0.83	42.77	8.24	0.45	53.47	104.93
5	Corn root tips	1.07	19	0	L	0.65	59.23	30.47	1.62	7.14	98.46
		1.08	19	0	D	0.65	62.84	32.55	1.52	4.85	101.76
		0.96	19	5	L	0.68	63.08	27.66	1.83	4.66	97.23
		0.96	19	5	D	0.63	54.56	30.75	1.72	8.66	95.69
6	Pea root tips	1.27	19	0	L	0.93	88.55	6.00	0.55	3.79	98.89
		1.22	19	0	D	0.92	81.20	6.08	0.59	4.58	92.45
		1.17	19	5	L	0.93	89.70	6.65	0.62	5.84	102.81
		1.13	19	5	D	0.92	85.93	7.19	0.61	6.22	99.95

in table III, about 83 % of the absorbed IAA-1-C^{14} was oxidized via oxidative decarboxylation and appeared in the respiratory CO_2. This result is in agreement with the work of Andreae and Van Ysselstein (1) that about 20 % of the IAA disappearing from solution could be accounted for as Salkowski reactive substances in the tissues. These substances were mainly IAA and indoleacetylaspartic acid. However, in the case of corn shoots no agreement was found with the work of Good et al (9) in which only 11.5 % of the loss of IAA from the medium can be accounted for by the reaction of the tissue extracts. It is possible that the age of the plants and the type of tissue organ used in this experiment may be responsible for this variation.

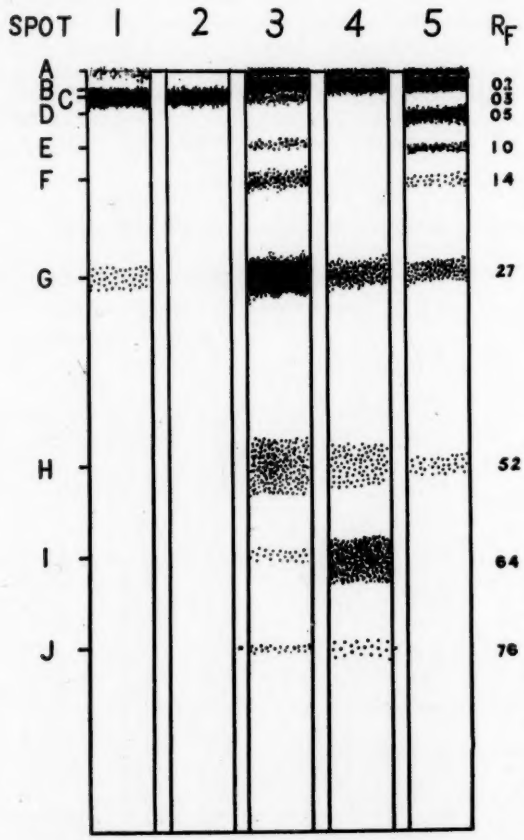


FIG. 1. Drawing of radioautographs of paper chromatograms showing the radioactive metabolites present in the alcohol extract of normal plant tissues which have been incubated in light with indoleacetic acid-1-C^{14}. 1. Pea shoots, 19 hrs incubation; 2. Pea roots, 19 hrs incubation; 3. Corn shoots, 19 hrs incubation; 4. Corn shoots, 6 hrs incubation; 5. Corn roots, 19 hrs incubation. The solvent system was: isopropyl alcohol, concentrated ammonium hydroxide, water (10 : 1 : 1 v/v).

Paper chromatographic data reveal the presence of 9 major radioactive metabolites in the alcohol extract of IAA-1-C^{14}-treated tissues. Typical radioautographs of paper chromatograms of alcohol extracts from normal plant tissues incubated in light with IAA-1-C^{14} solution are shown in figure 1. The distribution of radioactive carbon among various metabolites (as measured from the radioautographs by means of a densitometer) are given in table IV. From these percentages the rate of breakdown or incorporation of administered IAA in plant cells can be estimated, and some information can be gained about the nature of the metabolic pathways of IAA destruction.

In pea root tissue, 93 % of the absorbed IAA is destroyed via oxidative decarboxylation, and about 6 % is changed into indoleacetylaspartic acid. No free IAA-1-C^{14} was found in the pea root tissues under the experimental conditions. Including the free IAA, three radioactive compounds are found in the pea shoot extracts. The major fraction of the radioactivity is indoleacetylaspartic acid (R_f 0.03) which has been identified by co-chromatographic runs with an authentic sample from 2 different solvent systems. The amount of free IAA remaining in the tissues is small in both runs. Percentage-wise it is slightly more from the run having the shorter incubation period (6 hr) than from the run with the longer incubation period (19 hr). The 3rd radioactive compound A (R_f 0.00) has not been identified. The overall metabolic breakdown of IAA in pea shoot tissues is 79 to 87 % oxidative decarboxylation, 5 to 14 % formation of indoleacetylaspartic acid, and 1 to 2 % formation of compound A, which has not yet been identified. The free IAA remaining in pea shoots is less than 1% of the total IAA absorbed.

Apparently neither light nor 2,4-D treatment has any significant influence on the formation of these 2 radioactive metabolites. At present this evidence does not support the work of Galston (7) in which he concluded that prior treatment of the pea roots with some other auxin, such as 2,4-dichlorophenoxyacetic acid or α-naphthaleneacetic acid, interferes with IAA binding to the protein; the greater the concentration of analogue, the less IAA is bound. The data from the runs using pea shoot tissue reveal that 2,4-D treatment (10^{-4} M) increased slightly the ratio of indoleacetylaspartic acid to free IAA under both light and dark conditions. It is possible, however, that the bound IAA from pea roots measured in Galston's work is not indoleacetylaspartic acid. Moreover, since the experimental conditions (incubation time, IAA concentration in the medium, 2,4-D treatment, tissue organs used) are different in the two experiments, there is perhaps not sufficient justification for a comparison of results.

In the experiments with corn tissues, there are differences in the formation of metabolites between the runs having different incubation periods. Eight different radioactive metabolites were found in the extracts of corn shoots which were incubated for 19

TABLE IV

PAPER CHROMATOGRAPHIC STUDY OF THE FORMATION OF RADIOACTIVE IAA METABOLITES IN PLANT TISSUES

EXPERIMENT	PLANT TISSUES	EXPERIMENTAL CONDITIONS LIGHT OR DARK	2,4-D TREATMENT	TIME OF INCUBATION	DISTRIBUTION OF RADIOACTIVITY % OF TOTAL									
					A	B	C	D	E	F	G	H	I	J
			hr	hr	R_f 0	.02	.03	.05	.10	.14	.27	.52	.64	.76
1	Corn shoots	L	0	19	11.1	23.6	8.9		2.1	8.1	33.3	14.1	T	T
		D	0	19	10.7	18.6	10.5		2.2	7.4	38.8	12.1	T	T
		L	5	19	14.8	23.8	7.2		T	10.5	31.2	10.5	T	T
		D	5	19	17.7	20.6	11.8		T	7.9	27.3	14.8	T	T
2	Corn shoots	L	0	6	14.2	26.7					22.5	T	30.6	6.3
		D	0	6	10.5	22.4					26.6	T	32.7	8.0
		L	16	6	15.6	30.8					20.7	T	27.8	5.3
		D	16	6	16.2	30.5					20.0	T	27.9	5.7
3	Pea shoots	L	0	19	11.0		81.0				8.0			
		D	0	19	9.9		84.9				5.3			
		L	5	19	9.6		85.4				5.1			
		D	5	19	9.5		85.3				5.2			
4	Pea shoots	L	0	6	14.0		74.0				12.0			
		D	0	6	14.4		68.3				17.5			
		L	16	6	11.9		79.7				8.5			
		D	16	6	12.9		77.3				9.9			
5	Corn roots	L	0	19	4.5	57.7		29.9	4.5	T	5.7	T		T
		D	0	19	4.9	54.8		33.8	3.4	T	5.5	T		T
		L	5	19	6.9	69.7		19.0	T	1.6	6.2	T		T
		D	5	19	7.8	67.2		19.9	T	2.4	6.7	T		T
6	Pea roots	L	0	19			100							
		D	0	19			100							
		L	5	19			100							
		D	5	19			100							

hours. Their R_f values are 0.00, 0.02, 0.03, 0.10, 0.14, 0.27 (IAA), 0.52, 0.64, and 0.76. In the 6-hour incubation runs, 3 of these metabolites (R_f 0.03, 0.10, 0.14) are not formed, but 2 others appear in greater quantities on the radioautographs (R_f 0.64 and 0.76). Apparently the appearance of some metabolites is time dependent. Without a time course study, the metabolic sequence of conversion of the IAA molecule to other metabolites cannot be definitely determined. The metabolites formed in the first few hours of incubation may very likely change to other metabolites with an increase in incubation time.

Although the fundamental metabolic patterns of IAA breakdown are similar among the corn shoot tissue experiments, it is interesting to note that some quantitative variations in radioactive metabolite distribution (spots A and B) were observed in the runs carried on under the influence of light. As demonstrated by the average values of 3 separate runs, (the data of the 3rd run is not shown in table IV), the effect of light on the reactions with the non-treated shoots was an increase in the formation of both A and B by approximately 22 % and a decrease of the free IAA in the tissues by 24 %. The tissues receiving prior 2,4-D treatment did not exhibit these differences. Moreover, with the non-treated shoots, the presence of light increased the rate of oxidative decarboxylation by 22 to 25 %. Van Overbeek (14) in 1933 found that upon exposure of coleoptiles to weak light, the total amount of auxin remains the same, but that 85 % of it exists on the shaded side and only 15 % on the lighted side. The fact that the effect of light was less pronounced in the experiments conducted in this laboratory may be due to the use of much larger amounts of IAA than are natural to the plant. Moreover, some other factors such as the presence of other plant growth substances and their responses to light may also contribute to this difference. Nevertheless, the destruction of exogenous IAA in corn and pea shoots is greatly affected by light; therefore, it would seem that the destruction of endogenous IAA would be influenced similarly. Photo-destruction of IAA in response to light would be at least one of the mechanisms which are responsible for causing the asymmetric concentration of IAA. Treatment with 2,4-D may completely abolish the effect of light on the destruction of IAA, thus abolishing the phototropic response of plant organs.

There are 7 different radioactive metabolites in the extracts of corn root tissues. Chromatographic evidence indicates that there is no difference in IAA concentration between the light and dark runs of both normal and 2,4-D-treated tissues. This result is in agreement with the work of Audus and Thresh (2). However, pretreatment with 2,4-D caused a significant difference in the formation of metabolites B (R_f 0.02) and D (R_f 0.05). It increased the formation of B by 12 % (from 56 to 68 %) and correspondingly decreased the formation of D from 32 to 20 %. Metabolite D has been found only in corn root tissues and remains as yet unidentified. Preliminary work indicates that hydrolysis of metabolites A, B, C, D, E, and F with 1.5 *N* Ba $(OH)_2$ all produced radioactive IAA.

Summary

The in vitro studies of metabolic degradation of indoleacetic acid by normal and 2,4-D-treated plant tissues under conditions of light and darkness were carried out using carboxyl-C^{14}-labeled indoleacetic acid. The plant tissues were incubated in phosphate buffer containing 1.34×10^{-4} M IAA-1-C^{14} for various lengths of time. After incubation, the radioactivities of respiratory CO_2, of the alcohol extract of plant tissues, of the tissues after extraction, and of the ambient solution were determined. The metabolic products of IAA were identified chromatographically from the extracts.

Oxidative decarboxylation of IAA by corn and pea shoots was increased when the reaction was exposed to light. No influence of light was observed on the destruction of IAA by root tissues or by tissues pretreated with 10^{-4} M 2,4-D solution.

The major portion (80 to 92 %) of the absorbed IAA in pea tissues was metabolized via oxidative decarboxylation with the remainder changed to indoleacetylaspartic acid. The concentration of free radioactive IAA in pea tissues was small or negligible.

In contrast, 30 to 65 % of the absorbed IAA in corn tissues was oxidized and a variety of radioactive metabolites were identified chromatographically. Light affected the formation of 2 unidentified metabolites as well as the concentration of free IAA in normal corn shoots, but not in 2,4-D-treated ones. Formation of several radioactive metabolites depended upon the incubation time.

The participation of IAA destruction by light in phototropism is discussed.

The authors wish to express their appreciation to Dr. W. A. Andreae, Science Service Laboratory, Ontario, Canada, for the gift of indoleacetylaspartic acid.

Literature Cited

1. Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31: 235–240. 1956.
2. Audus, L. J. and Thresh, Ruth. The effects of synthetic growth-regulator treatment on the levels of free endogenous growth-substances in plants. Ann. Bot. 20: 439–459. 1956.
3. Boysen-Jensen, P. and Nielsen, N. Studien über die hormonalen Beziehungen zwischen Spitze und Basis der Avena Koleoptile. Planta 1: 321–331. 1926.
4. Bunning, E., Reisener, H. J. and Klebe, F. Versuche mit radioaktiver Indolylessigsäure zur Prüfung der sogenannten Ablenkung des Wuchshormonstromes durch Licht. Zeit Naturforsch. 113: 363–364. 1956.
5. Ching, T. M. and Fang, S. C. The redistribution of radioactivity in geotropically stimulated plants pretreated with radioactive indoleacetic acid. Physiol. Plantarum (In press.)
6. Fang, S. C. and Butts, J. S. Studies of carboxyl-C^{14}-labeled 3-indoleacetic acid in plants. Plant Physiol. 32: 253–259. 1957.
7. Galston, A. W. Some metabolic consequences of the administration of indoleacetic acid in plant cells. In: The Chemistry and Mode of Action of Plant Growth Substances, R. L. Wain and F. Wightman eds. Pp. 219–233. Butterworth Scientific Publications, London 1956.
8. Galston, A. W. and Baker, R. S. Studies on the physiology of light action. V. Photoinductive alteration of auxin metabolism. Amer. Jour. Bot. 40: 512–516. 1953.
9. Good, N. E., Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. II. Some products of the metabolism of exogenous indoleacetic acid in plant tissues. Plant Physiol. 31: 231–235. 1956.
10. Gordon, S. A. and Eib, M. Auxin transport in the phototropic response. Plant Physiol. 31 suppl. : xiv. 1956.
11. Henderson, J. H. M. and Peterson, L. L. Effect of dichlorophenoxyacetic acid on the geotropic and phototropic responses of the oat coleoptiles. Nature 179: 826–828. 1957.
12. Schrank, A. R. Plant tropisms. Ann. Rev. Plant Physiol. 1: 59–74. 1950.
13. Went, F. W. Wuchsstoff und Wachstum. Rec. trav. bot. néerl. 25: 1–116. 1928.
14. Van Overbeek, J. Wuchsstoff, Lichtwachstumsreaktion und Phototropismus bei Raphanus. Rec. trav. bot. néerl. 30: 537–626. 1933.

THE ELECTRON TRANSFER SYSTEM OF SKUNK CABBAGE MITOCHONDRIA[1, 2]

BRITTON CHANCE AND DAVID P. HACKETT[3]

THE JOHNSON FOUNDATION FOR MEDICAL PHYSICS, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PENNSYLVANIA, and DEPARTMENT OF BIOLOGY, UNIVERSITY OF BUFFALO, BUFFALO, NEW YORK

An unusual cytochrome b component in the spadix of Araceae remains oxidized in the presence of sufficient cyanide to reduce most of the cytochromes c—a—a_3 of the usual terminal respiratory enzyme sequence (1, 27). This pigment, called cytochrome b_7 by Bendall and Hill (1), is present in the mitochondrial fraction of the cell, and it has been suggested (1, 28) that it may account for the inhibitor-resistant spadix respiration by providing an alternate pathway of electron transport to oxygen. This possibility has been further examined with skunk cabbage, *Symplocarpus foetidus*, mitochondria (2, 3, 23, 24, 27).

In this paper we have reinvestigated the degree to which cytochromes a and c of the mitochondria of *Symplocarpus foetidus* become reduced upon the addition of azide and cyanide in order to determine whether they are completely disabled by these inhibitors or whether some residual activity remains, and thereby to test in detail the "excess oxidase" hypothesis (23, 25). It has further been possible to inhibit the oxidation of cytochrome b_7[4] of the mitochondria by treatment with 2-*n*-heptyl-4-hydroxyquinoline N-oxide (HOQNO) (19), showing that the b_7 component is much more closely related to the cytochrome c-a-a_3 system than was indicated by its insensitivity to urethane (1). Also included are studies of the effects of inhibitors upon the rates of oxygen and diphosophopyridine nucleotide (DPNH) utilization by the mitochondria. The nature of the electron transfer system of the mitochondria has been investigated, with special reference to the nature of the "c" and "b" components.

MATERIALS

Young skunk cabbage spadices were collected before the stamens had emerged and were stored in a cold room at 0° C. Flowers (10 g) were cut from the spadix and ground in a glass mortar with sand. The grinding medium (30 ml) contained 0.5 M sucrose, 0.05 M TRIS (tris hydroxymethylaminomethane) buffer (pH 7.0), and 0.01 M neutralized EDTA (ethylene diamine tetraacetate). The homogenate was strained through cheesecloth and centrifuged at 1,000 × G for 5 minutes, after which the mitochondrial fraction was sedimented at 10,000 × G for 20 minutes. The particles were washed by resuspending in 0.5 M sucrose-0.05 M TRIS and recentrifuging; the pellet was suspended in a small volume (3 ml) of the sucrose-TRIS medium. All operations were carried out in the cold room. For experimental observations, the final suspension was generally diluted about 10-fold in the reaction medium, which contained 0.5 M sucrose, 0.05 M TRIS, and 0.02 M phosphate (pH 7.0). The preparations contained roughly 15 mg protein per ml; the dilutions are given in the figure legends.

METHODS

A variety of spectroscopic and kinetic methods have been used in order to obtain sufficient data on these mitochondria to permit an evaluation of possible pathways of electron transport. For spectrophotometric recording, the double-beam apparatus has been used extensively (6). The extent of oxidation of cytochromes c and a in the presence of azide or cyanide can be accurately measured with negligible interference from cytochrome b_7. A convenient control for this is described below (p. 39).

In these experiments, the vibrating platinum microelectrode is used for measurement of respiratory activity (15). This method gives satisfactory results for DPNH and alpha-ketoglutarate (-c Kg) oxidase activities either in the absence of inhibitors or in the presence of such inhibitors as HOQNO or azide. Since cyanide "poisons" the electrode and causes a diminution of the electrode current that interferes with the calibration of the electrode sensitivity (20), we have refrained from including data on the respiratory activity measured by this method in the presence of cyanide.

The DPNH oxidase activity and the steady-state reduction of intramitochondrial reduced pyridine nucleotide (RPN) are readily measured by a fluorescence attachment to the double-beam spectrophotometer (11). The fluorescence of DPNH is excited by its absorption of light at 366 mμ. This light is obtained from a 100-watt, medium-pressure mercury lamp (General Electric (USA) A-H4) by means of a glass filter ("Eppendorf"-Netheler and Hinz-Hamburg). The fluorescence of DPNH is measured by a photomultiplier supplied with a Bausch and Lomb interference filter transmitting a band about 15 mμ, centered at approximately 450 mμ. There is, in addition, a Wratten

[1] Received July 7, 1958.

[2] This research was supported by grants to both authors from the National Science Foundation.

[3] Present address: Department of Biochemistry, University of California, Berkeley, California.

[4] The pigment which we have studied in this paper differs sharply from that described by Bendall and Hill in both its physical and its chemical properties (see Discussion). Since there is a possibility that a reconciliation of the experimental data can be made, we have refrained from naming a new cytochrome and use the term "cytochrome b_7" for the component of *Symplocarpus foetidus* that absorbs at 558 mμ.

2B filter to eliminate any leakage of the exciting light onto the photocell. The linearity of the fluorometric recording at the concentrations of DPNH employed is adequate for measurements of the activity. Non-linearity caused by absorption of the excitation light at higher concentrations of DPNH gave no difficulty, since a linear recording is necessary only at lower DPNH concentrations. Thus, the recording becomes more nearly linear as the added DPNH is oxidized.

The rapid-flow apparatus was utilized for studies of the reaction of the reduced cytochromes with oxygen. The form of apparatus employed is called the regenerative-flow apparatus (13, 17). The skunk cabbage mitochondrial suspension fills a large syringe (60 cc), and a smaller syringe (0.7 cc) is used to deliver the oxygenated buffer solution. The 2 syringes are driven simultaneously, and the 2 streams of fluid meet in the mixing chamber where oxygenation of the anaerobic mitochondrial suspension occurs. The oxidation reaction which ensues is observed in a 1 cm optical path chamber about 1 cm downstream from the mixing point. The times measured are roughly 10 milliseconds. The oxygenated solution is received in a 3rd syringe, and the flow may be continued until this 3rd syringe is filled, when its plunger reaches a stop. A few seconds after mixing, the added oxygen is expended by the mitochondrial suspension and the contents of the 3rd syringe become anaerobic. The fluid is then returned to the upper large syringe. At the same time, however, a stopcock connecting the 0.7 cc syringe to the mixing chamber is turned off, and this syringe is refilled from a separate supply of oxygenated medium. The apparatus is then ready for a 2nd experiment at a different wavelength setting. Thus the kinetics of oxidation of the various cytochromes are studied at appropriate wavelengths.

The spectra that are recorded in figures 1 to 4 are obtained with a split-beam recording spectrophotometer (6), with a special attachment for the studies at temperatures of liquid nitrogen (21).

Results

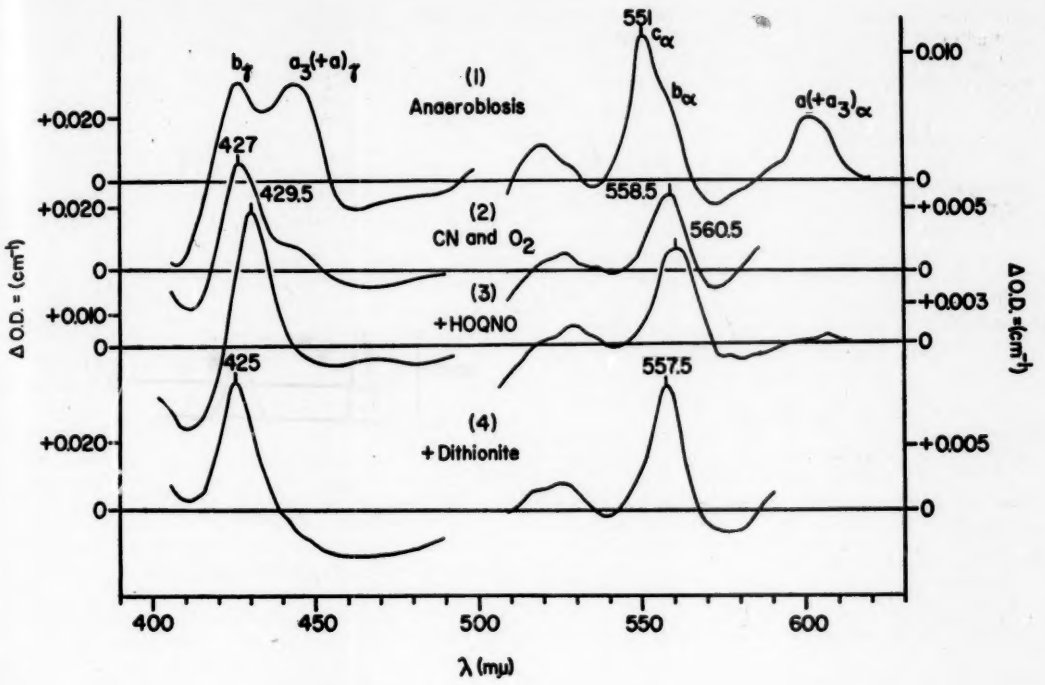
Spectroscopic Studies, Cytochrome b Components of the Mitochondria: The cytochrome components of the mitochondria can be most readily delineated by comparing the absorption spectra of 2 equivalent suspensions which have been subjected to different chemical treatments (9). For example, if substrate (αKg) is added to an actively respiring suspension, the dissolved oxygen is soon exhausted and the respiratory components become reduced. The difference spectrum obtained by comparing this suspension to one without substrate (oxidized) reveals the absorption bands of the respiratory chain components (fig 1, line 1). Absorption bands due to cytochromes of types a_3 (445 mμ), a (603 mμ), b_7 (558 mμ), c (551 mμ), and flavoprotein (460 mμ) are observed; these bands are in about the same positions as those reported by Hackett and Haas (24), although we find the c band at 551 mμ and the b_7 band with no measurable shoulder at 562 mμ. The b_7 band is displaced from that reported by Bendall and Hill (560 mμ), and the amount of b_7 is not so large as to completely dominate the spectrum, as is apparently the case with Arum (1).

Various chemical treatments can be used to reveal the cytochrome b components alone. The cytochromes that can be oxidized in the presence of cyanide are shown on line 2 of figure 1. Both samples in the split-beam spectrophotometer are allowed to become anaerobic and are then treated with cyanide. Oxygenation of the material in the reference cuvette gives the difference spectrum of the b_7 component with peaks at 558.5 and 427 mμ. These data show that the b_7 component can be satisfactorily measured by the double-beam method with one beam set at 558 mμ and with the other set at 575 mμ as a reference wavelength; the instrument then measures the difference of absorption between the two. The amounts of cytochromes a_3, a, and c oxidized under these conditions are difficult to evaluate. However, the shoulder on the Soret band of the b_7 component at about 445 mμ is caused by a combination of the Soret band of cytochrome a and the cyanide compound of cytochrome a_3. In the visible region, a rather broad absorption band in the region of 590 mμ is observed, only a portion of which could be due to the cytochrome a_3-cyanide compound.

A 2nd method for the delineation of the b component depends on the addition of HOQNO to the aerobic material (line 3). In this case, sharp absorption bands are seen at 560.5 and 429.5 mμ. None of the a or c components is observed. The displacement of these bands from those of line 2 suggests that material having an absorption peak at 566 mμ could be responsible for the shift of trace 3 to a longer wavelength. Such a component has been observed in heart muscle preparations to be formed upon the addition of HOQNO to cytochrome b (10).

Lastly, dithionite may be added to a substrate-reduced suspension in order to determine the nature of the components which are not reducible by electron transfer in the cytochrome chain (line 4). Two major absorption peaks are found: the α and γ bands are located at 557.5 and 425 mμ respectively. While the α band of this dithionite-reducible material suggests the same type of b component described above, the definite displacement of the Soret band to 425 mμ, together with the greater sharpness of these absorption bands, suggests that dithionite reduces a material distinct from that observed in traces 2 and 3. Interpretation of these data according to Bendall and Hill (1) suggests that the trace observed on line 2 consists of a mixture of cytochromes b_7 and b (a 557.5 and a 563 mμ component). This is not a satisfactory explanation for our material: subtraction of traces 2 and 4 gives, instead of a peak at 563 mμ, a small one at 567 mμ, a wavelength that does not correspond to a known type of cytochrome b.

The absorption bands of the cytochromes of types a and c can be clearly distinguished by treating both samples used in the split-beam spectrophotometer with substrate and HOQNO, in order to prevent the oxidation of the b component under aerobic conditions. The



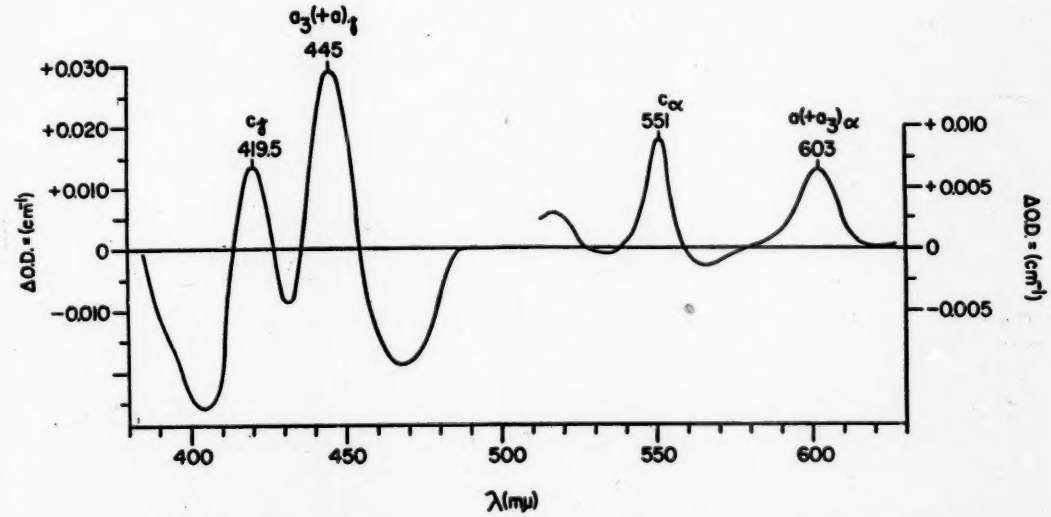


FIG. 1 (*top*). A study of the cytochrome b components of skunk cabbage mitochondria. By a series of chemical treatments (3, 23, 24), the cytochrome b components of the skunk cabbage mitochondria can be examined without interference from the cytochrome c—a—a_3 system. Line 1 is the reduced minus oxidized spectrum obtained with 4 mM $\sim$C Kg added to one sample of the material, the other sample containing only endogenous substrate. For line 2, both samples are treated with 4 mM $\sim$C Kg and 2 mM cyanide is added to one, after which both are aerated. Line 3 represents the difference between a sample treated with 4 μM HOQNO and a sample containing only endogenous substrate. Line 4 represents the effect of adding approximately 1 mg of dithionite to an anaerobic sample, the reference material being anaerobic in the presence of $\sim$C Kg. Volume 1.2 cc. Temperature 25° C. 0.7/2.5 mitochondrial dilution. (848 a).

FIG. 2 (*bottom*). A spectrum showing the absorption bands of the cytochrome c and a—a_3 components alone in skunk cabbage mitochondria. Both samples are treated with 4 μM HOQNO and 4 mM $\sim$C Kg and allowed to become anaerobic. One sample is then aerated; the resulting difference spectrum is plotted. Temperature 6° C. 0.7/2.5 mitochondrial dilution. (848 b).

difference between the reduced and oxidized samples, plotted in figure 2, shows the α bands of cytochromes a + a_3 and c at 603 and 551 mμ, and the Soret or γ bands at 444.5 and 419.5 mμ respectively. These values are very close to those for the mammalian components.

In summary, these spectroscopic data confirm the observation that the b component can be almost completely oxidized in the presence of cyanide, even though the a and c components are almost completely reduced. On the other hand, in the HOQNO-treated material, the b component can be almost completely reduced while the a and c components are almost completely oxidized. These results must be borne in mind when considering the relative insensitivity of the respiratory activity of the particles to inhibition by these 2 reagents.

TABLE I

SEQUENCE OF ABSORBANCY CHANGES OF ISOLATED MITOCHONDRIA (848)

COMPONENT	a-α	"b_7"	"c"	$(a_3)\gamma$	$c\gamma$	"$(b_7)\gamma$"
Absorbancy change in reduced-oxidized state, relative to cytochrome a	1	1.8	2.7	9	6	(see table II)

TABLE II

RELATIVE INTENSITIES OF ABSORPTION BANDS OF b COMPONENTS FROM DIFFERENCE SPECTRA OF SKUNK CABBAGE MITOCHONDRIA (848 a) (cf fig 1)

TREATMENT		HOQNO	CN + O_2	$Na_2S_2O_4$ TO ANAEROBIC MATERIAL
Peaks mμ	α	560.5	558.5	558
	γ	429.5	427	425.5
Ratio γ/α		8.3	8.1	3.9

RELATIVE VALUES OF ABSORBANCY CHANGES: The results summarized in table I indicate that the absorption caused by cytochrome a_3 relative to cytochrome a is greater than that for the mammalian respiratory chain (16). Such variations in the absorbancy ratios for cytochromes a and a_3 of the opposite sense have been found in microorganisms (4, 26). The absorbancy changes due to cytochromes of types b and c are somewhat higher, relative to cytochrome a, than in other types of mitochondria.

In table II we have indicated the ratio of the γ and α bands of the cytochrome b components revealed by the 3 chemical treatments of the mitochondria. In both the HOQNO and the cyanide treatments, the ratio is high (8:1) and similar to that expected of a cytochrome of type b. The dithionite treatment, however, gives a much smaller ratio (4 : 1). This result, together with the significant difference in the absorption maxima in lines 2 and 4 (fig 1), suggests that addition of dithionite to the substrate-reduced material affects a pigment that is not identical to the b_7 component.

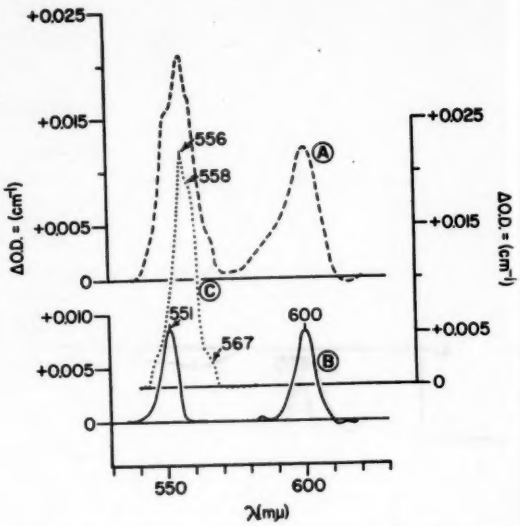


FIG. 3. Apparent absolute spectra of cytochrome components of skunk cabbage mitochondria at liquid N_2 temperatures. Trace A represents the absorption peaks that appear upon adding excess dithionite to the mitochondria. Traces B and C represent a separation of the components of trace A. Trace B represents those components that are reduced upon treatment of the mitochondria with 20 mM azide and 4 mM succinate. Trace C represents the difference spectrum in which the reference material consists of the material used to obtain trace B and the measured sample consists of the dithionite-treated material. Optical path 1 mm. 0.2/1.0 mitochondrial dilution. (848 c).

SPECTRA AT LIQUID NITROGEN TEMPERATURE: The cytochrome a and c components shown in trace B, figure 3, are the only 2 reduced bands observed when an aerobic suspension is treated with azide and succinate. The bands are located at 600 and 551 mμ respectively. The additional absorption caused by the b_7 component, indicated in trace A, is obtained by dithionite treatment. If the spectrum (B) is subtracted from (A), the difference spectrum is obtained (trace C). The major peak is located at 556 mμ with a large shoulder at 558 mμ and a very small one at 567 mμ. Since different b-type components are reduced by HOQNO and by subsequent dithionite treatment (see fig 1), caution must be exercised in interpreting the results obtained with dithionite. We shall make the assumption that the principal contribution to the low temperature spectrum is made by cytochrome b_7. Both the peak and the large shoulder (556 and 558 mμ) can be attributed to a single component, presumably b_7, which, in common with the b_2 and b_5 cytochromes, probably has a double α band at low temperatures

(12). The question of whether the split in the bands of cytochromes c, b_5, and b_2 corresponds to a mixture of two similar substances is apparently resolved by studies of purified cytochrome b_2 (12). No other b component, such as one with an absorption band at 560 mμ at low temperatures, is observed, in spite of the fact that Bendall and Hill (1) find one with a room-temperature peak at 563 mμ in Arum. The peak at 567 mμ at these temperatures does not correspond to any known b component. A comparison of these results with data presented in abstract form by Yocum and Bonner (27) shows agreement with our assignment of the 556 and 558 mμ bands to the b_7 component, to which they assign 555 and 558 mμ. However, they apparently find that a much larger proportion of a 552 mμ component is oxidized in the presence of cyanide. This difference and a difference in the position of the peak of the c component indicates that further experimentation at low temperatures is desirable. The peak at 551 mμ is significantly different from that of mammalian cytochrome c (548.5 mμ) or c_1 (554 mμ) (22), but it resembles that of a c_1-like component of fly sarcosomes (22). In the latter material, however, there is a c component of the usual type (548.3 mμ) which is apparently absent here. In the discussion that follows, the 551 mμ component will be termed cytochrome c for convenience only, but its difference from the cytochrome c of mammalian tissue is made clear by these spectra.

Respiratory Characteristics of the Mitochondria: It has already been demonstrated (23) that organic acids and DPNH are rapidly respired by skunk cabbage mitochondria. An example of the respiratory response to DPNH and α-Kg, as measured with an oxygen electrode, is given in figure 4, which

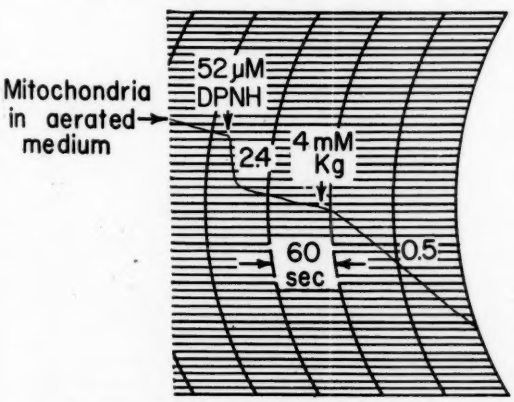


Fig. 4. Respiratory response of skunk cabbage mitochondrial suspension to a solution of DPNH (52 μM) and a solution of α-Kg (4 mM) as measured by the vibrating platinum electrode. The slopes of the trace are calculated for the steady-state respiratory activity in the presence of the two substrates and are respectively 2.4 μM O_2/sec and 0.5 μM O_2/sec as indicated on the graph. Temperature 26° C. The reaction medium as described in text. 0.3/2.5 mitochondrial dilution. (846 b).

Table III

Substrate Specificity of Skunk Cabbage Mitochondria (834)

Substrate	Endogenous	+ succinate	+ α-Kg	+ DPNH
Respiratory rate (μM O_2/sec)	0.06	0.7	1.0	5.0
$(TN)^*_a$ sec^{-1}	7	8	11	54

* Turnover number of cytochrome a

records an immediate and rapid response to DPNH and a delayed and slow response to α-Kg. Table III summarizes data on the respiratory activity of one preparation with several substrates. Succinate caused an increase in the respiratory activity, as did α-Kg. However, the principal activity of these mitochondria is clearly as a "DPNH oxidase" preparation, in agreement with previously reported results (23). Oxidase activity is expressed as the ratio of the respiratory rate to the cytochrome a absorption measured at 605 mμ with reference to 630 mμ. The ratio is termed $(K_4)_a$ (16). Since an approximate value for the extinction coefficient of cytochrome a is known, this value is converted into a turnover number ($(TN)_a$) by multiplying by 4 to convert the oxygen rate to an iron rate and by 16 to convert the absorbancy change to a millimolar iron basis, and is divided by 10^3 to convert the millimolar iron concentration to micromolar units. These turnover numbers indicate a DPNH oxidase activity that is intermediate between those obtainable under optimal conditions with heart muscle sarcosomes and with flight muscle sarcosomes (see (14)).

Other skunk cabbage preparations containing considerably smaller amounts of endogenous substrate had a similar ratio of DPNH oxidase to α-Kg oxidase activities. In a few preparations, DPNH oxidase activity resulting from successive additions of small concentrations of DPNH (approximately 20 μM) was observed to decrease rapidly between the 1st and 2nd addition. On the other hand, most preparations show a decrease of activity of only a few percent when measured in this manner.

Steady States, General Properties of Mitochondria: In order to examine the respiratory mechanisms in greater detail, steady states of the various components were followed spectrophotometrically in relation to the respiratory activity of the preparation. The record shown in figure 5 illustrates the type of experimentation and demonstrates the responses of the b_7 and RPN components, as well as of the oxygen uptake, to substrate and inhibitors. The mitochondria are initially diluted in the reaction medium, and, if they are adequately washed, little or no change of oxygen concentration is recorded by the vibrating platinum electrode. At room temperature, addition of 4 mM α-Kg causes a fairly rapid increase in both the reduced cytochrome b_7 and RPN, as indicated by the downward deflection of the traces. It

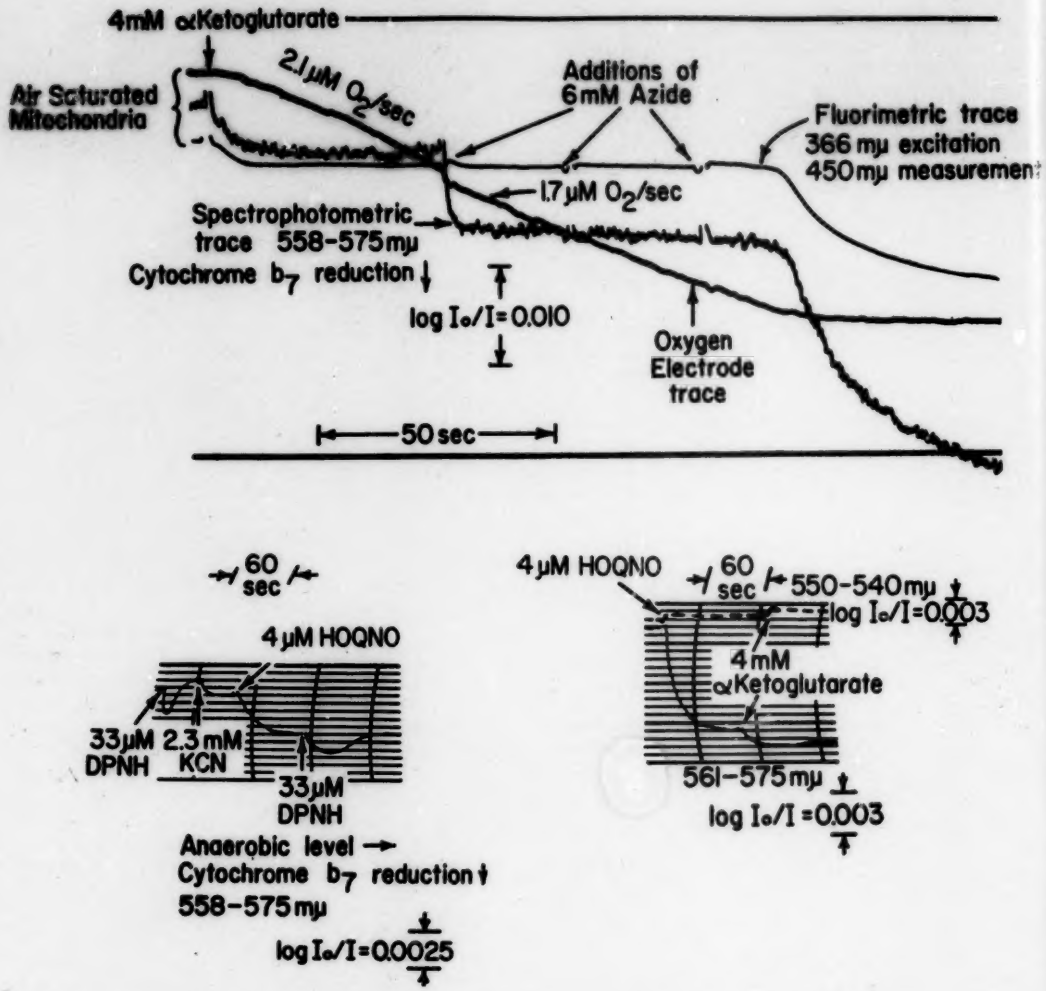


FIG. 5 (*top*). The effects of substrates and inhibitors upon respiratory activity and steady states of cytochrome b_7 and pyridine nucleotide components of the skunk cabbage mitochondria. The vibrating platinum electrode is used to measure the respiratory response to addition of αKg and additions of azide, so marked on figure. The double-beam spectrophotometer is used to measure absorbancy due to increasing reduction of cytochrome b_7 as indicated by a downward deflection of the spectrophotometric trace which corresponds to increasing absorption at 558 relative to 575 mμ. Pyridine nucleotide is measured by a fluorescence attachment to the double-beam spectrophotometer which provides 366 mμ excitation and a filter for measurement at 450 mμ. A downward deflection corresponds to an increasing fluorescent intensity. Temperature 25° C. 0.4/2.5 mitochondrial dilution. (833–3).

FIG. 6 (*bottom, left*). Effects of DPNH, cyanide, and HOQNO upon the steady state of the cytochrome b_7 component. This record is similar to that of figure 5 except that only the spectrophotometric trace is provided. The sequential additions of DPNH, KCN, and HOQNO are indicated on the graph. Increasing reduction of cytochrome b_7 is indicated by a downward deflection of the trace and the level obtained in anaerobiosis is marked by the arrow. Temperature 26° C. pH 6.5. 0.3/2.5 mitochondrial dilution. (837 a).

FIG. 7 (*bottom, right*). Demonstration of the lack of interference of reduction of the b component upon the measurement of the steady state of the c component in skunk cabbage mitochondria. Two experiments are superimposed here. In one, absorbancy changes due to the c component are measured at 550 mμ with reference to 540 mμ (dashed line). In the other, large absorbancy changes of the b component are measured at 561 mμ with respect to 575 mμ and are caused by the addition of 4 μM HOQNO followed by 4 mM αKg. 0.2/2.5 mitochondrial dilution. (845 b).

should be noted, however, that the reaction is not instantaneous and that the steady-state reduction of RPN and of cytochrome is reached only after some seconds. At about that time, the induction period in the initiation of the respiratory activity is terminated and the respiratory rate is nearly established at its maximum rate of 2.1 μM O_2/second. Addition of 6mM azide causes an abrupt increase in the reduction of the b_7 component, but it has no significant effect on the RPN component as measured fluorometrically. The platinum microelectrode trace shows a slight irregularity caused by the addition of the inhibitor, after which the respiratory activity decreases to 1.7 μM O_2/second. The 2nd and 3rd additions of 6 mM azide cause no changes in the steady-state levels of the pigments or in the rate of respiration. Upon exhaustion of the oxygen dissolved in the suspension, the reduction of both pigments begins and then continues for a considerable interval after the platinum electrode indicates that the respiration has come essentially to a standstill.

The responses of the b_7 component to additions of DPNH and HOQNO are illustrated in figure 6. The recording begins with the mitochondria diluted in the reaction medium and containing only endogenous substrate. Addition of 33 μM DPNH causes an abrupt reduction of the b_7 component followed by its oxidation when the added DPNH has been utilized. Addition of 2.3 mM potassium cyanide causes a slight reduction of the b_7 component, presumably due to the inhibition of cytochrome oxidase activity. A further large reduction of the b_7 component is obtained upon addition of 4 μM HOQNO. When calculated as a percentage of the total absorbancy change obtained in anaerobiosis (line marked "anaerobic"), cyanide causes a 14 % reduction while HOQNO plus cyanide causes a reduction of 49 %. The addition of DPNH to the cyanide-and-HOQNO inhibited system slowly increases the reduction of the b_7 component to 65 %; this is followed by its oxidation to the previous level when the added DPNH has been expended.

Studies of this type have been carried out at wavelengths appropriate to a number of respiratory components in order to determine their response to various inhibitors. In each case, the percentage reduction of the component is computed assuming that the transition from the aerobic, substrate-free state to the anaerobic state corresponds to a 100 % change. We ignore the additional reduction caused by dithionite, since the spectra illustrated above (fig 1) show that a pigment other than the b_7 component is involved. In some cases we have taken the liberty of measuring the b component not only at 558 mμ but also at 562 mμ in order to determine whether significant differences are to be found at these wavelengths. We offer, however, no evidence that there is, in addition to the b_7 component, a b cytochrome which absorbs at 562 mμ and which can with certainty be distinguished from the b_7 component in these preparations.

Interference from Cytochrome b_7: It is important to know whether measurements of the changes in the steady state of cytochromes a and c may be confused by overlapping changes in the steady state of cytochrome b_7. For example, the extent of oxidation in the presence of azide or cyanide is measured by recording the increment of deflection that occurs when the inhibitor-treated suspension becomes anaerobic, the spectrophotometer being set at wavelengths appropriate to cytochromes a and c. At the same time, however, large absorbancy changes occur at wavelengths absorbed by the cytochrome b_7 component which could conceivably interfere. This possibility has been tested by following the changes which accompany the large change in absorbancy of the b_7 component caused by the addition of HOQNO to an aerobic suspension. Under these conditions, a negligible absorbancy change at wavelengths appropriate to cytochromes c and a would be expected, if the reduction of the b_7 component does not interfere. Figure 7 shows 2 traces, one recorded at wavelengths appropriate to the b_7 component (561 to 575 mμ), the other recorded at wavelengths appropriate to the c component (550 to 540 mμ). The mitochondrial suspensions are initially aerobic and contain only the endogenous substrate. Addition of 4 μM HOQNO causes an immediate, large (67 %) reduction of the b_7 component. At the wavelengths appropriate for recording the c component, there is a slight disturbance at the moment HOQNO is added. The disturbance is of opposite sign to that which could be mistaken for cytochrome c reduction, and its magnitude is about 4 %. Similar studies at wavelengths appropriate to cytochrome a (605 to 630 mμ) show a similar small disturbance. We may conclude, therefore, that measurements of the residual oxidation of cytochromes a and c in the presence of excess cyanide and azide are not confused by absorbancy changes due to cytochrome b_7. The slight disturbance described above would cause the amounts of cytochromes a and c remaining oxidized in the presence of inhibitors to be underestimated rather than overestimated.

Effect of Azide: Azide is convenient for simultaneous recording of the effect of an inhibitor upon respiration and upon the steady states of cytochromes c and a. The sensitivity of the platinum microelectrode is only slightly affected by additions of azide, whereas the effect of cyanide on its sensitivity is so large that this technique cannot be used. Figure 8 A presents a summary of steady-state and respiratory measurements, based on experiments of the type shown in figure 5; the steady-state reduction was calculated as described above. Results were obtained for cytochromes a, c, b_7, and flavoprotein by spectrophotometric methods and for RPN by the fluorescence method.

The results show that the respiratory activity is depressed 14 % by 6 mM azide and that further additions of azide cause no further inhibition. The steady states of cytochromes a and c reflect this respiratory inhibition. An 86 % reduction of cytochrome a is caused by the 1st addition of azide, but no further decrease occurs thereafter. The c component shows a less abrupt approach to the maximal reduction (80 %) obtainable in the presence of azide. The flavoprotein component does not show a distinct plateau at these

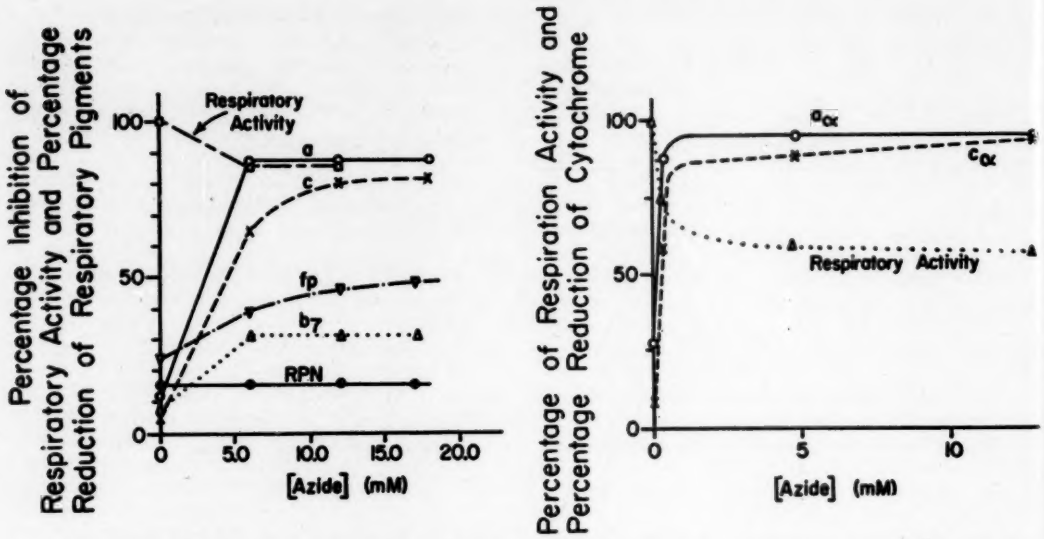


FIG. 8. Effect of azide upon respiratory activity and extent of reduction of respiratory components. Fig 8 A. -c Kg as substrate. Cytochromes a, c, and b_7 are measured at wavelengths indicated in the text. Flavoprotein is measured at 465 mμ with reference to 500 mμ. RPN is measured fluorometrically. The respiratory activity is measured by the platinum microelectrode. Temperature 25° C. 0.4/2.5 mitochondrial dilution. (833). Fig. 8 B. DPNH plus -c Kg as substrate. 0.2/2.5 mitochondrial dilution. Other conditions as in figure 8 A. (833, 845 a).

values of azide concentration. The response of the b_7 component is similar to that of the a component, although the maximal reduction obtained is only about 30 %. The intramitochondrial RPN, measured fluorometrically, shows no change of reduction. It is apparent from these results that the failure of azide to cause more than 14 % inhibition of the -c Kg oxidase activity is not due to the absence of excess inhibitor.

The extent to which the cytochromes are activated in the absence of inhibition depends on the nature of the respiratory substrate. For example, the percentage reduction of cytochrome a, as indicated in figure 8 A for 0 azide, is about 10 %. The addition of DPNH or DPNH + -cKg causes a larger reduction of this component (26 %) and a 5-fold greater turnover number. We have, therefore, examined the effects of azide on respiration and the cytochromes when DPNH and -cKg are the substrates (fig 8 B). Under these conditions, the respiratory activity is much more sensitive to low azide concentrations. Half maximal inhibition occurs at a concentration of about 0.5 mM azide, and 44 % reduction of the respiratory rate is obtained with 12 mM azide. The reduction of cytochrome a is similarly abrupt and reaches a plateau of 95 % reduction at 5 mM azide. The reduction of cytochrome c increases somewhat less abruptly and reaches 93 % at 12 mM azide.

In summary, figures 8 A and 8 B show that the degree of inhibition of respiratory activity caused by a given concentration of inhibitor depends critically upon the extent of activation by the particular substrate: the greater the activation, the greater the inhibition. This finding agrees with the earlier report (23) that organic acid oxidation is less sensitive to cyanide than is the DPNH oxidase activity. This conclusion also applies to the extent of reduction of cytochromes a and c in the steady state: the greater the activity, the greater the reduction. In neither case, however, are these cytochromes completely reduced; a sufficient amount of the oxidized cytochrome remains to raise questions as to whether any alternate pathway for electron transfer in the azide-inhibited system needs to be considered at this time.

EFFECT OF CYANIDE: In accordance with the results obtained with azide, figure 9 shows the increasing reduction of cytochrome c as a function of the cyanide concentration, using endogenous substrate. There is an abrupt increase of reduction at lower cyanide concentrations, and thereafter the reduction approaches 90 % at 4 mM cyanide. The DPNH oxidase activity of this preparation showed some instability (see above) and therefore a somewhat less abrupt increase of reduction of cytochrome c with increasing cyanide concentration. Higher concentrations of cyanide were not used because of its well-known side effects. The conclusion reached on the basis of these data is the same: measurable amounts of the oxidized cytochrome are present in a system containing large amounts of the inhibitor.

EFFECT OF AZIDE AND HOQNO: As indicated by the spectroscopic data, HOQNO causes a large reduction of the cytochrome b component. Table IV illustrates the effect of this inhibitor upon the steady states and the respiratory activity. First, it is seen that

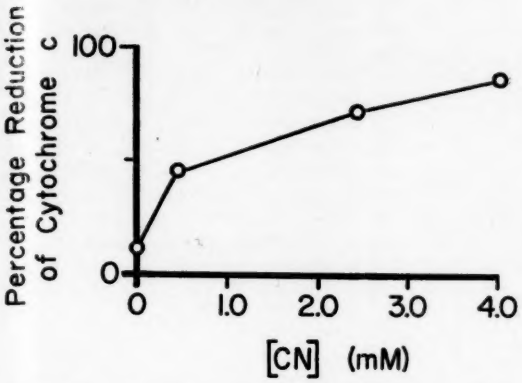


FIG. 9. The effect of cyanide upon the percentage reduction of cytochrome c, DPNH as substrate. Cytochrome c is measured at 550 mμ with respect to 540 mμ. At least 1 minute was allowed for equilibration of the cyanide effect and steady states were measured after a sufficient number of additions of DPNH so that a reasonable degree of completion of the cyanide inhibition had been obtained. Temperature 25° C. 0.2/2.5 mitochondrial dilution. (843).

TABLE IV

EFFECT OF AZIDE AND HOQNO UPON STEADY STATES AND RESPIRATION WITH DPNH AND ₵Kg AS SUBSTRATES (836)

SUBSTRATE	INHIBITOR	% INHIBITION OF DPNH OXIDATION	% REDUCTION OF b_7
DPNH	azide	21	38
DPNH + ₵Kg	"	21	...
DPNH	azide + HOQNO	32	61
DPNH + ₵Kg	"	58	72

4 μM HOQNO causes an appreciable inhibition of respiration: a value of 32 % is obtained for DPNH oxidation. In accordance with the spectroscopic data, the reduction of cytochrome b_7 increases to 61 %. When both substrates are used, the inhibition of DPNH oxidation is about 58 % and the reduction of cytochrome b_7 exceeds 70 %. These data show not only that HOQNO increases the steady-state reduction of b_7, but also that it inhibits electron flow through the system. This agrees with the earlier report (23) that antimycin A can inhibit organic acid oxidations and the DPNH oxidase of isolated mitochondria. The results with HOQNO are, furthermore, qualitatively similar to those obtained with azide and cyanide, in that a large reduction of a cytochrome component can be observed while the electron flow through the whole system is only partially inhibited.

TABLE V

EFFECT OF CYANIDE AND HOQNO ON STEADY STATES AND DPNH OXIDATION (837)

SUBSTRATE	CN mM	HOQNO μM	% REDUCTION OF c	% REDUCTION OF b_7	% REDUCTION OF DPNH* OXIDATION
DPNH	2.4	0	95	...	71
DPNH	2.4	4	...	57	76

* Values not corrected for spontaneous decrease of DPNH oxidase activity.

Effects caused by cyanide and HOQNO are similar to those shown in table IV and are illustrated in table V. The addition of HOQNO to the cyanide-inhibited cytochrome a_3 system gives a 57 % reduction of cytochrome b_7. Here, the extent of respiratory inhibition caused by HOQNO is less than was obtained in table III, possibly because of slight differences in the mitochondria. Nevertheless the results are in qualitative agreement, especially with respect to increased reduction of b_7.

EFFECT OF HOQNO ON CYTOCHROME c: It is further possible to show that the flow of reducing equivalents to the cytochrome system is inhibited by HOQNO treatment of the mitochondria (fig 10). The trace begins with the mitochondria suspended in aerated reaction medium. Addition of ₵Kg accelerates the respiration after the usual lag period and causes

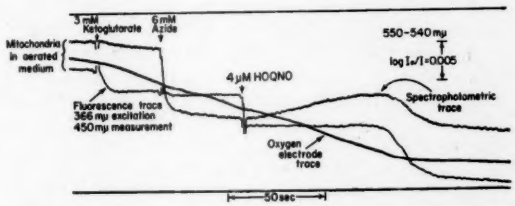


FIG. 10. Spectrophotometric evidence that an oxidation of cytochrome c accompanies the reduction of cytochrome b_7 upon treatment of mitochondria with HOQNO. This record combines respiratory, spectrophotometric, and fluorometric records similar to that of figure 5 (833–3), and an explanation of the traces is given there. In this case the spectrophotometric trace records cytochrome c oxidation as an upward deflection. Temperature 26° C. 0.3/2.5 mitochondrial dilution. (830 a–7).

increased reduction of DPNH as indicated by the fluorescence trace. Only a small reduction of cytochrome c occurs in this interval. Addition of 6 mM azide causes a large reduction of cytochrome c as indicated by the abrupt downward deflection of the spectrophotometric trace. A very small change in the fluorescence trace is observed. After about half a minute, the oxygen trace resumes the previous rate. Addition of 4 μM HOQNO causes, by its own fluo-

rescence, an abrupt downward deflection of the fluorescence trace which separate calibrations show involves little or no change in the oxidation-reduction level of this component. As the HOQNO inhibits electron transfer in the cytochrome system, an upward deflection of the spectrophotometric trace is observed which corresponds to an oxidation of cytochrome c. The experiment is terminated by anaerobiosis, which causes complete reduction of cytochrome c and DPNH.

This experiment renders very unlikely the possibility that the reduction of cytochrome b_7 by HOQNO is a consequence of inhibition of an "alternate pathway" for b_7 oxidation not involving the cytochrome c–a–a_3 system. In summary, the increasing reduction of the b_7 component upon the addition of the terminal respiratory inhibitors such as cyanide and azide together with its increased reduction in the presence of HOQNO makes it clear that the reduced form of this component accepts oxidizing equivalents from the cytochrome c–a–a_3 system.

Effect of Amytal: The skunk cabbage mitochondria are, surprisingly enough, insensitive to concentrations of amytal which completely and rapidly block electron transfer in the mammalian mitochondrial suspensions (2 mM) (7). It is difficult to say whether this is a consequence of the general unresponsiveness of the skunk cabbage material to respiratory inhibitors or whether a special amytal-insensitive DPNH-flavoprotein relationship exists in this system.

Kinetic Studies, Kinetics of Reduction: In order to study the kinetics of reduction of the cytochrome components of the mitochondrial preparations, a number of special experimental conditions must be satisfied. Since it is important to begin the experiment in the substrate-free condition, the mitochondria were subjected to an additional washing procedure. DPNH was used as the substrate in order to avoid the complications which would result from the delayed initiation of α-Kg oxidation. The reaction rates were reduced by working at 0° C to 2° C. To conserve material, the experiments were carried out by rapidly adding DPNH to the mitochondrial suspensions in an open cuvette rather than in the rapid-flow apparatus.

The nature of the experimental results is indicated in figure 11, where the addition of 15 μM DPNH is recorded on the right by an abrupt downward deflection of the fluorometric trace. As indicated by the linear upward deflection of the trace, this substrate is rapidly utilized in an approximately zero-order reaction at the rate of 0.3 μM DPNH/second. The simultaneous spectrophotometric trace, which records absorbancy changes at wavelengths appropriate to the b_7 component, shows an abrupt downward deflection corresponding to a rapid reduction of this component. The rate of this reaction, measured at as near zero time as is possible with this technique, corresponds to 0.17 μM Fe/second. This calculation assumes that the molecular extinction coefficient for b_7 at 558 mμ is 20 $cm^{-1} \times mM^{-1}$. After most of the DPNH has been utilized, the cytochrome component is slowly reoxidized. As shown in the left-hand portion of the figure, DPNH addition causes a similar abrupt reduction of the cytochrome c component; in this case, the rate is 0.09 μM Fe/second.

A summary of the rates of reduction of cytochromes b and c is given in table VI. In the absence of cyanide, these rates, calculated from the initial slope of the reaction upon the addition of a solution of 20 μM DPNH, are in the order $b_7 > c$. It must be emphasized that these kinetics represent approaches to the steady state, since they were measured in the absence of a terminal inhibitor. Nevertheless the sequence of rates is consistent with the idea that the b components are active in electron transfer to the cytochrome c–a–a_3 system.

Table VI

Kinetics of Reduction of Cytochrome Components Caused by DPNH Addition at 2° C (846)

Wavelengths (mμ)		Measurement	550	558	562
		Reference	540	575	575
"Initial rate" μM Fe/sec	[HCN]	0	0.09	0.17	0.10
		4 mM	0.07	0.10	0.05

If this experiment is repeated in the presence of 4 mM cyanide, the same qualitative result is obtained, although all the rates are, for an unknown reason, slightly slower. At this high cyanide concentration, cytochrome c is considerably reduced prior to the addition of DPNH because the washed preparation contains endogenous substrate.

Kinetics of Oxidation: In order to record the kinetics of oxidation of the mitochondrial cytochromes, it was necessary to use the regenerative flow apparatus, as was found to be the case with mitochondria prepared from liver (17). In this apparatus, a low concentration of oxygen (3 μM) is rapidly mixed with the mitochondrial suspension and the kinetics of oxidation of the cytochrome components are measured spectrophotometrically, using the double-beam spectrophotometer as in the studies of the kinetics of reduction described above. Figure 12 shows the reaction of the α-Kg-reduced material with oxygen. The left portion records the experiment initiated with cytochrome c in the completely reduced state. Upon initiating the flow, as indicated by the downward deflection of the flow velocity trace at the top of the figure, and after an initial transient, there is a rapid upward deflection of the lower trace, indicating a decrease of absorbancy due to oxidation of cytochrome c. This trace rises to the point labeled "oxidized at 9 ms" which is roughly half-way between the completely reduced state (beginning of the trace) and the steady-state oxidized condition (maximum upward excursion of the trace). The calibration of the flow velocity trace indicates that this corresponds to a time of 9 milliseconds. When the flow stops, the maximum upward deflection is obtained and persists until the added oxygen has been depleted, at which time the cytochrome becomes reduced.

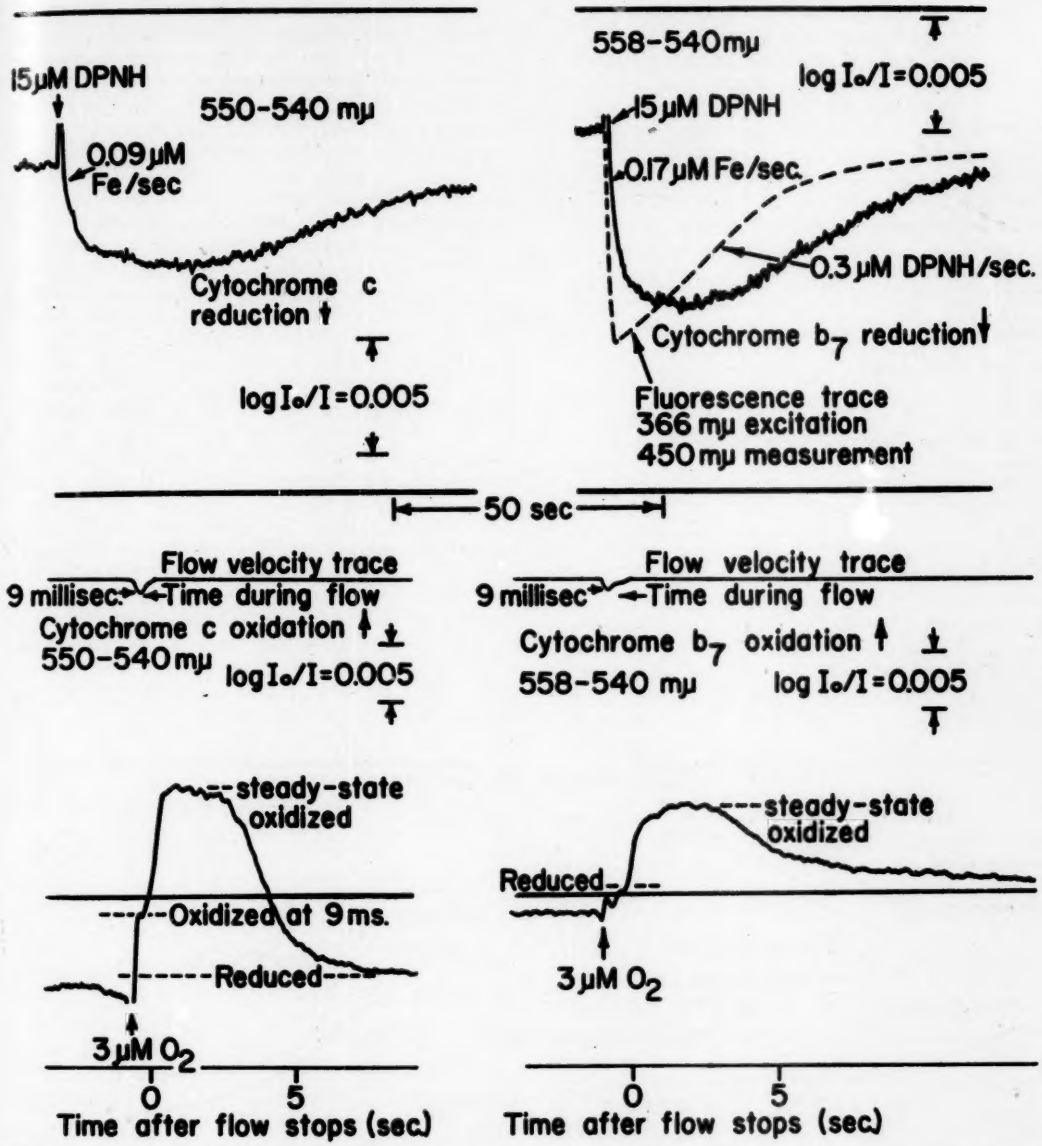


FIG. 11 (*top*). Experimental data on kinetics of reduction of cytochromes of the mitochondria at 2° C. The right-hand record indicates the reduction of cytochrome b_7 together with the fluorescence trace indicating the utilization of the added DPNH. The left-hand record represents similar experiments in which the reduction of cytochrome c is measured. A downward deflection of the traces represents a reduction. The mitochondrial preparation was subjected to an additional washing in order to remove endogenous substrate. 0.3/2.5 mitochondrial dilution. (846–3).

FIG. 12 (*bottom*). Experimental records of the kinetics of oxidation of cytochrome c and cytochrome b_7 as recorded in the regenerative flow apparatus. The wavelengths for recording cytochrome kinetics are those used previously and an upward deflection of the traces indicates an oxidation. The shortest time reached during the flow is marked by an arrow on the top trace, and the corresponding oxidation of cytochrome is emphasized by the dashed line "oxidized at 9 ms." for cytochrome c. The steady-state oxidized level and the average value for the "reduced" level are indicated for both records. It should be noted that the initial and final values for the reduced levels are not identical due to the slight dilution of the preparation that occurs during the discharge of the oxygenated buffer solution into the mitochondrial suspension. Thus the "reduced" levels represent the average of the 2 values. Temperature 4° C. 20-fold mitochondrial dilution. (842 b).

The right-hand trace records the corresponding kinetics of the b_7 component. Here the trace rises very little during the flow of liquid through the observation tube, reaching a level no higher than the calibration obtained after the added oxygen has been exhausted. It is clear that no appreciable oxidation of cytochrome b_7 occurs at 9 milliseconds and that its reaction is slow enough that the trace after the flow has stopped has a measurable slope. A summary of data obtained under these conditions appears in table VII.

TABLE VII

KINETICS OF OXIDATION OF CYTOCHROMES AT 4° C (842 b)

WAVELENGTHS (mμ)		MEASUREMENT	550	558	562
		REFERENCE	540	575	575
"Initial rate" μM Fe/sec	[HCN]	0	8	0.33	0.25
		4 mM	...	0.18	0.21

In a 2nd series of experiments, the oxidizability of the b components was determined in the presence of 3 mM cyanide. The kinetic data show the rate of oxidation of the b_7 component to be roughly half its value in the absence of cyanide. In the presence of this concentration of cyanide, the steady-state reduction of the c components is so great that the absorbancy change on oxygenation of the mitochondria is too small to measure accurately in the rapid-flow apparatus.

CORRELATION OF REDUCTION TIMES: In investigations of phosphorylating mitochondria prepared from liver, it was found that reduction of all the cytochromes proceeds to completion at about the same rate in the aerobic-anaerobic transition (the time difference between the earliest (a_3) and the latest (b) is small). This is not the case in nonphosphorylating Keilin-Hartree heart muscle particles (5). Since a small time difference affords a criterion for inclusion of cytochromes in the respiratory pathway, it is desirable to apply it to the skunk cabbage material. Figure 13 shows simultaneous recordings of the kinetics of oxygen utilization and the reduction of DPNH and the 2 cytochromes in the aerobic-anaerobic transition. The time at which the extent of reduction of the respiratory components is compared is marked "$O_2 \rightarrow 0$" (intersection of the straight lines tangent to the two straight portions of the platinum electrode trace), although, as a matter of fact, a small amount of oxygen still remains in the suspension. At that time, however, we are able to compare accurately the extent of reduction of the pigments; the results are summarized in table VIII. In accordance with the data obtained with other mitochondria, the c component is about 50 % reduced at "$O_2 \rightarrow 0$", and RPN is 27 % and 31 % reduced in the 2 records. However, cytochrome b, recorded here at 562 mμ, is only 18 % reduced, and a similarly slow reaction is recorded for b_7 at 558 mμ. While this discrepancy is small, it suggests that some of the b and b_7 components may not be fully active in electron transfer.

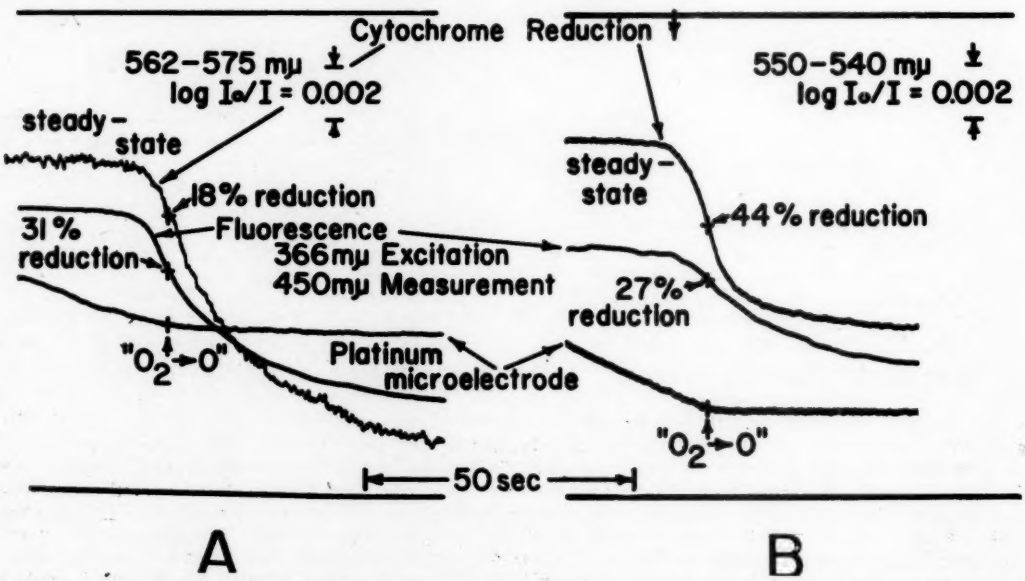


FIG. 13 A comparison of the kinetics of reduction of the cytochromes in the aerobic-anaerobic transition. In fig 13 A the cytochrome b component is recorded together with RPN. In fig 13 B the cytochrome c component and RPN are recorded. The point "$O_2 \rightarrow 0$" is the intersection of the extrapolated slopes of the platinum electrode traces, which serves as an accurately defined index at which to measure the percentage reduction of the pigments involved. (833–2, 847 b).

TABLE VIII

EXTENT OF REDUCTION OF RESPIRATORY ENZYMES IN THE AEROBIC-ANAEROBIC TRANSITION (833 to 847 b)

		% INCREASED REDUCTION AT "$O_2 = 0$"		
COMPONENT		c	"b"	RPN
Expt	*Inhibitors*			
847 b	none	44	...	27
833–2	none	...	18	31
830 a–7	6 mM azide 4 μM HOQNO	20	...	50

INTERACTION BETWEEN PHOSPHORYLATION AND ELECTRON TRANSFER: In the experiments described so far, the reactions were carried out with mitochondria capable of oxidative phosphorylation in the medium in which they were studied (24). We have, therefore, investigated the effect of adenosine diphosphate (ADP) upon the rate of electron transfer, since it has a marked effect on mitochondria isolated from the mammalian tissues. The increase of respiratory rate following the addition of ADP was found to be negligible compared with that measured in mammalian mitochondria, but it could, nevertheless, be demonstrated. A similar small increase of respiratory activity was obtained upon adding 80 μM dibromophenol (DBP). Spectroscopic observations indicate that the cytochrome b component was approximately 15 % oxidized upon the addition of ADP or DBP (measured at 558 mμ). In the presence of 0.4 mM cyanide, the change in the steady state of the b component caused by adding ADP was considerably less. The control that ADP exerted over electron transfer in these mitochondria was insufficient for a clear demonstration of the localization of sites of oxidative phosphorylation, as was possible in the mammalian mitochondria. The qualitative observations that small changes of respiratory rate and steady-state cytochrome reduction occur upon addition of ADP to the mitochondrial system suggest that a similar type of oxidative phosphorylation mechanism exists in these mitochondria, but further studies are required to confirm this supposition.

DISCUSSION

NATURE OF THE ELECTRON TRANSFER CHAIN: The presence in Aroid spadix tissue of a cytochrome system resembling the c–a–a_3 system of the mammalian tissue has been established by previous work (1, 3, 24, 25, 28). Nevertheless, there are certain quantitative differences in the properties of the components which cannot be ignored. For example, the absorption band due to the cytochrome a component appears to be smaller than in typical mammalian material. Thus, the average of the ratio of absorbancy change due to the a_3 component to that due to the a component approaches 9 : 1, whereas 7 : 1 is a usual value for the mammalian system (16, 26). A determination of the ratio of cytochrome c oxidase activity to absorbancy change at 630 mμ in the difference spectrum showed the value for skunk cabbage mitochondria to be roughly 10-fold greater than the values for most mammalian preparations (Lucile Smith, personal communication). However, the significance of any determination of cytochrome oxidase activity in particulate preparations depends upon a number of factors that are difficult to control.

At room temperature, the c component has absorption bands with peaks at 551 and 419 mμ which are very close to those of the mammalian cytochrome c. At low temperatures, there is no appreciable shift of the α band of this pigment, nor does it split into two components. We can put forward little evidence for more than one type of c component in the mitochondria, and this component may differ somewhat from that of the mammalian system. For this reason, we would refer to this component as c_{551}, thereby distinguishing it from the c_1 analogue found in fly sarcosomes (22), which we here designate $c_{1(551)}$.

The chief interest in this respiratory chain has centered around the cytochrome b-type components. On the basis of spectroscopic and oxidation-reduction potential studies of Arum preparations, Bendall and Hill (1) described 2 b components with absorption bands at 560 and 563 mμ. Our studies of the skunk cabbage mitochondria show some qualitative differences. First, we find that the α and γ bands of the component remaining oxidized in the presence of cyanide or azide lie at 558.5 and 427 mμ respectively. We consider significant the displacement from 560 to 558.5 mμ (see footnote 4). In liquid N_2, the 558.5 mμ peak probably splits into two peaks at 556 and 558 mμ, designated α_1 and α_2 respectively. The positions of these absorption bands and the splitting at low temperatures are not characteristic of the cytochrome b of the mammalian respiratory chain, but they are characteristic of non-respiratory cytochromes such as b_2 and b_5 (12). The positions of the absorption bands of these cytochromes are summarized in table IX. It is clear from these data that the α bands of

TABLE IX

COMPARISON OF LOW-TEMPERATURE SPECTRA OF CYTOCHROMES OF TYPE b (848 c)

CYTOCHROME	α_1	α_2
b_2	553.1	557.1
b_5	553.7	558.4
b_7	556.5	558.5
b*	560.5	...

* c—a—a_3 system

these 3 pigments have 2 absorption peaks, and that in each case the larger peak lies at the shorter wavelength. The interval between these 2 peaks is 2 mμ for the b_7 pigment and 4 mμ for the b_2 and b_5 pigments; otherwise their spectroscopic properties appear similar.

The role of this atypical cytochrome b has been examined with the aid of HOQNO, which gives rise to an absorption peak at 560 mμ when added to aerobic mitochondria at room temperature. This peak ap-

parently has the characteristics of the b_7 component even though it is shifted slightly toward longer wavelengths by the HOQNO treatment, as is the case with a portion of the b pigment of heart muscle preparation (10). This observation disagrees with the report of Bendall and Hill that urethane, which usually causes reduction of cytochromes of type b, has no effect upon the b_7 compound of Arum preparations (1). It is possible that more intensive treatment of the Arum material with urethane might have revealed the effects observed here with the skunk cabbage mitochondria. From our experiments with HOQNO, we conclude that cytochrome b_7 depends to a considerable extent upon the cytochrome a–c system for its oxidation. This fact must be carefully considered in theories that postulate the b_7 component as a portion of a separate terminal oxidase system that is insensitive to cyanide and azide.

The data presented here may also help to explain the observation that the b_7 component, which has an oxidation-reduction potential of —0.03, is only half reduced by substrate, the remainder being reducible only by dithionite (1). We find that the material reduced by dithionite has somewhat different light absorption characteristics from that reduced by substrate. Not only are the α and γ bands at different positions, but the ratio of their intensities is significantly different. It is suggested that, in addition to the b_7 component, there is another component, reducible only by dithionite, which does not participate in electron transfer and whose properties require further investigation. It is well known that many preparations from mammalian tissues show the presence of dithionite-reducible material which does not participate in electron transfer (10).

While Bendall and Hill's data suggest the presence of a "typical b" component with an absorption band at 563 mμ in the Arum material, evidence in favor of such a pigment in the skunk cabbage mitochondria is almost nonexistent. The low temperature data do not show a sharp absorption band at 560.5 mμ, as is characteristic of the mammalian pigment, but instead they show a small band at 566 to 567 mμ which may represent a rather different b component. These results suggest that the identification of the b component of the mammalian electron transfer system with a component in the skunk cabbage mitochondria, and possibly also in the Arum material, is premature.

A flavin component is identified in the difference spectrum, and as far as can be ascertained it behaves similarly to that observed in mammalian systems. However, the large absorption bands of carotenoids in this region of the spectrum make accurate measurements difficult. RPN is readily measured by fluorescence change in the mitochondria, and its behavior towards added αKg suggests that the fluorescent material is the DPNH of the respiratory chain.

On the basis of these data, we propose, in equation 1, a pathway of electron transfer in these mitochondria to consist of a sequence of cytochromes, flavoproteins, and pyridine nucleotides analogous to those of the mammalian system. There are, however, some unknown factors and some qualitative differences.

$$\alpha\text{Kg} \rightarrow \text{DPNH} \rightarrow \text{fp} \rightarrow b_7 \rightarrow c_{551} \rightarrow a \rightarrow a_3 \rightarrow O_2 \quad (1)$$

(with b? above the fp → b_7 step, and O_2 below the fp → b_7 step)

The b_7 component appears to fulfill satisfactorily two of three criteria for establishing its ability to transfer electrons in the cytochrome chain: 1st, the kinetics of its oxidation following the addition of oxygen to the anaerobic mitochondria and 2nd, the kinetics of its reduction following the addition of DPNH to the aerobic material appear to be consistent with the position in electron transfer indicated in equation 1. It cannot be denied, however, that the rate at which the b_7 component approaches the anaerobic steady state when oxygen is removed due to respiration of the mitochondria suggests either that a portion of the b_7 is less active or that an alternate pathway functions at the level of flavoprotein and cytochrome b_7.

The possible effectiveness of these alternate pathways is evaluated below:

Possible Explanations of Insensitivity to Inhibitors: The simplest explanation of the rapid rate of oxygen utilization in spite of very large steady-state reduction levels of the cytochromes obtained by treatment with inhibitors is that the inhibition of cytochrome action by the various reagents is incomplete. This excess oxidase hypothesis has been carefully examined by Yocum and Hackett (28) and Hackett and Haas (24), whose calculations suggest that an extraordinarily high oxidase-dehydrogenase activity would be required to explain the insensitivity to inhibitors on this basis. This possibility was rejected by Bendall and Hill (1) because they were unable to detect any oxidized cytochrome a or c in the aerobic, cyanide-treated Arum material. Our measurements have demonstrated the presence of small amounts of oxidized cytochromes a and c in skunk cabbage mitochondria in the presence of large concentrations of inhibitors. There are also indications that the cytochrome oxidase turnover number is rather high. Thus it would appear that the excess oxidase hypothesis cannot yet be entirely eliminated.

The excess oxidase hypothesis is one statement of the little-understood property of the electron-transfer system to show large changes of the steady state, in portions of the system where the concentration of the components is not rate limiting, and at the same time to show no appreciable change in total electron-transfer rate. This we have termed the "cushioning effect" of the cytochrome chain (28). Certain configurations of the concentrations and reaction velocity constants are more favorable to a large "cushioning effect" than others, and physical arguments can indicate clearly an appropriate configuration. For example, the respiratory chain of the mammalian mito-

chondria in the absence of ADP shows a considerable insensitivity to the inhibition of electron transfer by azide (8). This is because the rate-limiting step is between RPN and flavoprotein, the RPN being nearly completely reduced and the flavoprotein being largely oxidized. Cytochrome a_3 is very nearly completely oxidized in the absence of inhibitor and, in spite of the addition of sufficient azide to cause its nearly complete reduction in the steady state, there is enough of the reduced, uninhibited oxidase to carry on the electron transfer process with negligible inhibition. A qualitative indication of the probable insensitivity of a respiratory chain to the addition of the inhibitors would be indicated by the extent of oxidation of the respiratory components in the absence of that inhibitor. In the skunk cabbage mitochondria, we find rather small reductions of the respiratory components (see fig 8 B) in the presence of $\mathfrak{c}$Kg. Thus these steady-state values suggest a system appropriately proportioned for a "cushioning effect." Such an effect is to be expected with inhibitors that are dissociated from the terminal oxidase at a rate comparable to the turnover number of the oxidase in electron transfer activity. This certainly applies to azide and perhaps also to cyanide, although detailed studies of the reaction kinetics are incomplete.

An "excess oxidase" situation might also arise as the result of an inaccessibility of certain portions of the mitochondria to the inhibitors carbon monoxide, cyanide, and azide (25). This possibility is supported by Bonner's finding that insensitivity to azide may be diminished by treatment of the mitochondria at low pH (2). This observation is difficult to reconcile with the fact that the intact system responds readily to oxygen but is resistant to carbon monoxide. Thus, more than a simple inaccessibility to inhibitors is involved in the Aroid respiratory chain.

The 2nd possibility that has been considered (1, 24, 28) is that these tissues possess an alternate, inhibitor-resistant pathway of electron transport to oxygen through cytochrome b. This hypothesis, that some cytochrome b is oxidized directly or through a cyanide-insensitive system is considerably weakened by a number of experimental observations reported here. First, kinetic studies show that the rate of oxidation of cytochrome b_7 is actually slightly diminished by 3 mM cyanide and that its steady-state reduction increases in the presence of azide. Thus, the pathway involved is not completely insensitive to these inhibitors. Second, it is found that a large increase in the reduction of the b_7 component is caused by HOQNO treatment. This suggests that the pathway for the oxidation of b_7 is through the cytochrome c–a–a_3 chain. The fact that antimycin A also inhibits is also significant because of the high specificity of this substance for electron transfer in this chain.

In attempting to distinguish between the excess-oxidase and the alternate-pathway hypotheses, one must take into account two important facts: 1) both cytochromes a and c remain somewhat oxidized in the presence of high concentrations of azide and cyanide, and 2) the b_7 component, the oxidation of which is considerably inhibited by HOQNO, nevertheless remains somewhat oxidized in the presence of high concentrations of this reagent. Thus, evidence for an alternate-pathway hypothesis would be rendered equivocal by the presence of small amounts of the oxidized forms of cytochromes a, b_7, and c which have not been proved incapable of transferring electrons at a rate sufficient to explain the oxidase activity. In summary, although it is difficult to prove the excess-oxidase hypothesis, it cannot be discarded at the present time, and until it is discarded consideration of alternate-pathway mechanisms must remain secondary.

Some properties of the hypothetical alternate pathway may be worth considering. On the basis of tissue respiration studies, it was pointed out earlier (28) that the "alternate oxidase" must have a high oxygen affinity. This has been confirmed with isolated mitochondria by following the rate at which respiratory activity falls to zero in the aerobic-anaerobic transition: the Michaelis affinity (K_M) for the azide-resistant $\mathfrak{c}$Kg oxidase activity is in the order of 10^{-6} M (as indicated by the platinum microelectrode). It is therefore unlikely that flavoprotein affords an alternate pathway. The possibility that the spadix contains reducible organic substrate which is present in large quantities or is sufficiently autoxidizable to sustain a cyanide-insensitive oxidation of the cytochromes or flavin components is also unlikely, since successive and exhaustive washings of the mitochondria do not appear to diminish their insensitivity to inhibitors. It is of interest that some preparations of the skunk cabbage mitochondria showed a rapidly diminishing DPNH oxidase activity towards successive additions of substrate, but that this did not appear uniformly in all preparations. These considerations do not leave us with a plausible alternate pathway.

While the b_7, c, and a components show variations in their steady-state levels caused by the inhibitors that appear to be inconsistent with the constant respiratory rate, the steady-state level of intramitochondrial RPN shows a relative insensitivity to inhibitors that is in reasonable accord with the respiratory activity, at least in the presence of $\mathfrak{c}$Kg. It would seem, therefore, that any alternate pathway or any rate-limiting step involved in an excess-oxidase theory would operate not between b_7 and O_2 but between b_7 and RPN (equation 1). Flavoprotein also shows increasing reduction under conditions of relative constancy of respiration. However, the accuracy of such observations is somewhat less than that for the other components, because of the large absorption bands of carotenoids present in the skunk cabbage preparation. If the present observations are correct, it would appear that the rate-limiting step or branch of the chain might be at the level of RPN itself, although further experiments on this point are required.

It has been reported (24) that even in the absence of any inhibition of oxygen uptake, cyanide can inhibit oxidative phosphorylation by skunk cabbage mitochondria. This is a remarkable effect, since we have not yet been able to show in isolated mammalian

mitochondria that changes in the steady-state levels of respiratory components decrease the phosphate/oxygen (P/O) ratio. Nevertheless the systems are different and the P/O value may be dependent upon the steady-state levels in the spadix. To explain such a dependency, we imply that the free energy change available for phosphorylation depends upon the steady-state concentrations of the carriers; the large reduction of c and a in the presence of cyanide inhibits phosphorylation in this region of the chain. Alternatively, the decrease of P/O value could be attributed to the activation of a nonphosphorylating, inhibitor-resistant electron transport pathway. The observation that the phosphorylation efficiency is reduced roughly 50 % by cyanide suggested that 2 of the 4 phosphorylating steps are blocked. Since the uninhibited sites would presumably be at the levels of substrate and RPN, this observation agrees with the above conclusion that the unknown feature of this respiratory chain is near the substrate side in the sequence of electron transfer components. On the other hand, the b_7 oxidase hypothesis would predict 3 inhibitor-resistant steps, one each at the substrate, RPN, and cytochrome b levels. However, the available phosphorylation data do not permit an unequivocal decision on this point.

Summary

Spectroscopic observations of skunk cabbage mitochondria show the presence of an atypical cytochrome b (b_7) which has α-c and γ absorption peaks at 558 and 427 mμ, remains largely oxidized in the presence of cyanide and azide, and is largely reduced by HOQNO and antimycin A. In addition, the mitochondria posses flavoprotein, cytochrome c, and cytochrome a–a_3 components, as well as dithionite-reducible, cytochrome-like material that does not participate in electron transfer. The cytochrome c component differs from that of the mammalian system in the location of its absorption maxima and may be designated c_{551}. Evidence in favor of c_1 and b components analogous to those of the mammalian respiratory chain is lacking at present. The significant difference between the pattern of respiratory carriers of these mitochondria and of those of the mammalian system suggests that a different response to inhibitors is not unexpected.

Although the respiratory activity of skunk cabbage mitochondria is relatively insensitive to inhibitors of the mammalian system (cyanide, azide, and HOQNO), considerable inhibition can be demonstrated, especially at the higher levels of activity obtained with DPNH as substrate. Spectroscopic observations of the steady-state levels show that even though cytochromes a, c, and the b_7 component are more reduced in the presence of these inhibitors, they remain oxidized to a measurable extent. Thus both the respiratory and the spectroscopic studies suggest that the function of the cytochrome c–a–a_3 system may not be assumed to be completely inhibited with any type or concentration of inhibitor so far studied. It would seem, therefore, premature to give serious consideration to alternate pathways for electron transfer in this system until the inhibition of the known pathway can be shown to be complete.

The use of HOQNO permits a more detailed study of the possible role of the b_7 component in electron transfer. It is found that the oxidation of this component depends upon electron transfer through the cytochrome c–a–a_3 system: it can be demonstrated not only that cytochrome c is an oxidant for the b_7 component but also that the b_7 component is a reductant for the cytochrome c component. Kinetic studies show that the rate of reduction of the b_7 component by DPNH and the kinetics of its oxidation in the uninhibited system are consistent with its role in transfer through the cytochrome c–a–a_3 system. In fact, it is only a discrepancy in relative rates of reduction of the cytochromes in the aerobic-anaerobic transition that raises any question of the function of b_7 as a component of a single cytochrome chain of the skunk cabbage mitochondria.

Literature Cited

1. Bendall, D. S. and Hill, R. Cytochrome components in the spadix of *Arum maculatum*. New Phytol. 55: 206–212. 1956.
2. Bonner, W. D., Jr. Properties of particulate preparations from *Symplocarpus foetidus*. Plant Physiol. 32 suppl. : xiv. 1957.
3. Bonner, W. D., Jr. and Yocum, C. S. Spectroscopic and enzymatic observations on the spadix of skunk cabbage. Plant Physiol. 31 Suppl.: xli. 1956.
4. Chance, B. Respiratory pigments of metabolising cells. Federation Proc. 11: 196. 1952.
5. Chance, B. Spectra and reaction kinetics of respiratory pigments of homogenized and intact cells. Nature 169: 215–221. 1952.
6. Chance, B. Spectrophotometry of intracellular respiratory pigments. Science 120: 767–775. 1954.
7. Chance, B. Interaction of adenosinediphosphate with the respiratory chain. In: Enzymes, units of biological structure and function, O. H. Gaebler, ed. Pp. 447–463. Academic Press, New York 1956.
8. Chance, B. Cellular oxygen requirements. Federation Proc. 16: 671–680. 1957.
9. Chance, B. Techniques for the assay of the respiratory enzymes. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. IV Pp. 273–329. Academic Press, New York 1957.
10. Chance, B. The kinetics and inhibition of cytochrome components of the succinic oxidase system. III. Cytochrome b. Jour. Biol. Chem. 233: 1223-1229 1958.
11. Chance, B., Conrad, H. and Legallais, V. Simultaneous fluorometric and spectrophotometric measurements of reaction kinetics of bound pyridine nucleotide in mitochondria. Program and abstracts of the Biophysical Society. Pp. 44–45. Massachusetts Institute of Technology, Cambridge 1958.
12. Chance, B., Klingenberg, M. and Boeri, E. Cytochrome b_2 and b_5 systems. Federation Proc. 15: 231. 1956.
13. Chance, B. and Legallais, V. Regeneration and recirculation of reactants in the rapid-flow apparatus Part 2.—Practical designs. Discussions Faraday Soc. 17: 123–128. 1954.

14. CHANCE, B. and SACKTOR, B. Respiratory metabolism of insect flight muscle. II. Kinetics of respiratory enzymes in flight muscle sarcosomes. Arch. Biochem. Biophys. 76: 509–531. 1958.
15. CHANCE, B. and WILLIAMS, G. R. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. Jour. Biol. Chem. 217: 383–393. 1955.
16. CHANCE, B. and WILLIAMS, G. R. Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. Jour. Biol. Chem. 217: 395–407. 1955.
17. CHANCE, B. and WILLIAMS, G. R. Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. Jour. Biol. Chem. 217: 429–438. 1955.
18. CHANCE, B. and WILLIAMS, G. R. Respiratory enzymes in oxidative phosphorylation. VI. The effects of adenosine diphosphate on azide-treated mitochondria. Jour. Biol. Chem. 221: 477–489. 1956.
19. CORNFORTH, J. W. and JAMES, A. T. Structure of a naturally occurring antagonist of dihydrostreptomycin. Biochem. Jour. 63: 124–130. 1956.
20. DAVIES, P. W. and BRINK, F. Microelectrodes for measuring local oxygen tension in animal tissues. Rev. Sci. Instr. 13: 524–533. 1942.
21. ESTABROOK, R. W. The low temperature spectra of hemoproteins. I. Apparatus and its application to a study of cytochrome c. Jour. Biol. Chem. 223: 781–794. 1956.
22. ESTABROOK, R. W. and SACKTOR, B. The respiratory metabolism of insect flight muscle. III. Low-temperature spectra of the cytochromes of flight muscle sarcosomes. Arch. Biochem. Biophys. 76: 532–545. 1958.
23. HACKETT, D. P. Respiratory mechanisms in the Aroid spadix. Jour. Exptl. Bot. 8: 157–171. 1957.
24. HACKETT, D. P. and HAAS, D. W. Oxidative phosphorylation and functional cytochromes in skunk cabbage mitochondria. Plant Physiol. 33: 27–32. 1958.
25. SIMON, E. W. Succinoxidase and cytochrome oxidase in mitochondria from the spadix of Arum. Jour. Exptl. Bot. 8: 20–35. 1957.
26. SMITH, L. Bacterial cytochromes. Bacteriol. Rev. 18: 106–130. 1954.
27. YOCUM, C. S. and BONNER, W. D., JR. Electron transport in *Symplocarpus foetidus*. Proceedings of the American Society of Plant Physiologists and the Physiological Section, Botanical Society of America, Indiana University, August 24–28. Pp. vi–vii. 1958.
28. YOCUM, C. S. and HACKETT, D. P. Participation of cytochromes in the respiration of the Aroid spadix. Plant Physiol. 32: 186-191. 1957.

Note added in proof: Bendall (Bendall, D. S. Cytochromes and some respiratory enzymes in mitochondria from the spadix of *Arum maculatum*. Biochem. Jour. 70: 381–390. 1958) has determined the low temperature spectra of the dithionite-reduced components of Arum mitochondria and finds 600, 558, and 549 $m\mu$ bands which he attributes to cytochromes a, b_7, and c. Succinate and antimycin-a treatment show bands at 563, 558, and 553 $m\mu$ which may be attributed to b, b_7, and c_1. In agreement with these results and those of Yocum and Bonner (27) on skunk cabbage, no cytochrome b component with a peak at 560 $m\mu$ is observed. The absence of the prominent band at 556 $m\mu$ in Bendall's spectra is remarkable and suggests a significant difference between the "b_7" components of the lily and the skunk cabbage.

THE RESPONSE OF THE YOUNG TOMATO PLANT TO A BRIEF PERIOD OF WATER SHORTAGE. IV. EFFECTS OF WATER STRESS ON THE RIBONUCLEIC ACID METABOLISM OF TOMATO LEAVES [1, 2]

C. T. GATES[2] AND JAMES BONNER

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

It is known from previous studies (1) that when young tomato plants are subjected to water stress the growth of the plants is checked. Leaves of different ages are differently sensitive to such stress. Thus, young leaves suffer the greatest absolute growth check but recover most rapidly. Older leaves are checked less but recover from water stress more slowly (2).

The check in growth induced by water shortage in the leaves of young tomato plants is accompanied by depression in net accumulation in the leaves of nitrogen and phosphorus. Severely water stressed older leaves may even lose nitrogen and phosphorus. In fact (3) the first metabolic effect of stress which has as yet been detected is decreased accumulation of total phosphorus.

This paper concerns further observations on the effect of moisture stress on the phosphorus metabolism of young tomato plants. It will be shown that moisture stress causes decreased accumulation of ribonucleic acid (RNA) and may in fact block RNA accumulation entirely. In addition, the moisture stressed leaf retains ability to incorporate P^{32} into RNA. The blocked net synthesis of RNA in water stressed plants may therefore reside in an increased rate of destruction of the material.

METHODS

The standard inbred line of tomato (*Lycopersicon esculentum* Mill var. Pearson) used in past investigations of this series was also used for the present ex-

[1] Received July 10, 1958.
[2] Report of work supported in part by a grant from the Division of Natural Sciences and Agriculture, Rockefeller Foundation.
[3] Permanent address, Commonwealth Scientific and Industrial Research Organization, Griffith, N. S. W., Australia.

periments. In all experiments, unless otherwise noted, treatment consisted of a single period of moisture stress applied to the whole plant. Only the effects of the whole plant moisture stress on the behavior of the leaves are considered in this paper. At harvest, the laminae of the leaves were separated from the petioles at the junction with the petiolules. The leaves are identified as to age by numbers, the oldest being number 1. One experiment, that for which results are given in figures 2 and 3, was carried on in an ordinary greenhouse. All other experiments were conducted in a controlled environment chamber.

The cultural details of the greenhouse experiment are as follows: The plants were grown in 32 oz. cans each containing 850 g Jondaryan loam (7) having 180 ppm phosphorus as a dilute acid soluble P_2O_5 (method of Truog, 1930). One nutrient application was made on day 0, at which time 30 ppm phosphorus and 14 ppm nitrogen (oven dry weight of soil basis) were supplied, and one on day 24 at which time 16 ppm nitrogen was supplied.

The experiment included 2 treatments, a control, in which soil moisture was maintained as nearly as possible at field capacity, and a water stress treatment, in which the plants were not watered after day 31. At this stage, the laminae of the 3rd pair of leaves were approximately one third of full size. Each treatment was applied to 3 groups of plants, each comprising 4 blocks of 5 plants, chosen at random along the length of the greenhouse bench, so as to control positional variance. Of the 3 groups of 20 plants collected at each harvest, 2 were used for analyses of fresh material including RNA, and 1 for estimation of dry matter, total phosphorus and total nitrogen. Harvests were made on days 31, 33, 35 and 38.

Total leaf phosphorus was estimated by Gerritz's (4) modification of the Zinzadze procedure, and total nitrogen by Kjeldahl. In the experiment of figures 2 and 3, total nucleic acid was estimated spectrophotometrically with 5 % perchloric acid extracts made at 70° C, following a modification of the method of Ogur and Rosen (5).

Plants of the remaining experiments were grown in plastic pots containing a mixture of gravel, 75 %, and vermiculite, 25 % by volume. These were watered as required with deionized water and received a daily application (sufficient to bring the pot to above field capacity) of Hoagland's nutrient solution. The plants were used for treatment when 28 to 30 days of age. Each experiment included 2 treatments. In one, the control treatment, daily watering was continued. The moisture-stress treatment consisted merely of withholding water during the experiment. Treatments were allotted at random within size groups determined visually. Each harvest of each treatment consisted in general of 10 plants, collected in 2 groups of 5 each.

In all experiments other than that of figures 2 and 3, RNA was estimated spectrophotometrically by extraction, after removal of pigments and soluble nucleotides, with 0.33 *N* potassium hydroxide following the method of Schmidt and Thannhauser (6), followed by adjustment of the pH of the extract to pH 1.5 to 2.0. DNA as extracted from the residue with 5 % perchloric acid at 70° C and also measured spectrophotometrically.

The parent RNA nucleotide mixture obtained by the KOH hydrolysis and extraction was further purified by separation of the nucleotides by ascending chromatography in Wyatt's (8) solvent, or by paper electrophoresis in 0.05 *N* ammonium acetate buffer adjusted to pH 4.5 with acetic acid. The absorption spectra of the original RNA hydrolysate and of the nucleotides separated by chromatography are presented in figure 1.

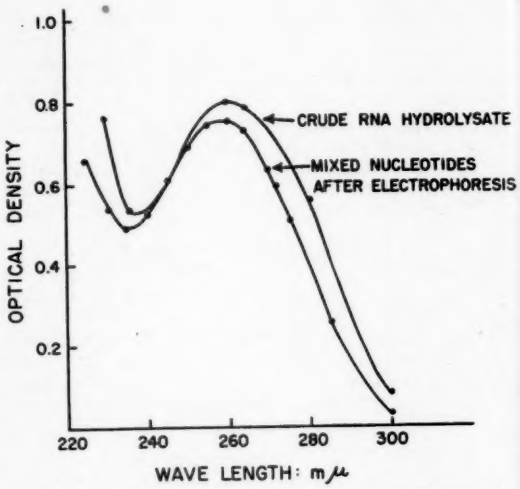


FIG. 1. Absorption spectra for typical RNA hydrolysate preparations. Spectra from experiment of table III. Recovery of RNA nucleotides after electrophoresis was 91 % in this case.

Recovery of nucleotides after purification by chromatography varied between 80 and 100 %; after purification by electrophoresis between 86 and 100 %. The specific radioactivity of the nucleotides purified by chromatography was approximately one half that of the parent RNA hydrolysate. Purification by electrophoresis yielded nucleotides of specific activity approximately 75 % of that of the parent RNA nucleotide mixture. Conclusions concerning RNA synthesis and turnover based on measurements of the parent RNA nucleotide mixture were in all cases identical with the conclusions based on measurements of the purified RNA nucleotides.

In experiments in which P^{32}-labeled phosphate was used, radioactivity was measured with a Geiger tube equipped with a micromil window and operated in an atmosphere of Q gas. Counts per minute minus background (cpm) at infinite sample thinness are given in the tables below.

In experiments in which P^{32}-labeled phosphate was supplied to plants, it was always provided in a solution 10^{-5} M with respect to P^{31} phosphate.

Experimental Results

Experiments with Soil Moisture Tension Induced Water Stress: In an initial experiment plants were grown in the greenhouse until 31 days of age. At this time 1 cycle of increasing moisture stress was given to 1 replicated set of plants. The control series was kept at near field capacity throughout. In the moisture stress treatment the soil became gradually drier during the 7 days of the experiment. The soil moisture did not drop below the permanent wilting percentage although it approximated this value closely at the final harvest as is shown in figure 2. The depressing effect of moisture stress on dry weight increments, particularly in the case of the 3rd pair of laminae, is clearly evident in the data of table I. The inhibition of relative growth rate (grams increment/100 grams whole plant dry weight/day) by moisture stress is clearly evident in the data of figure 2.

The effects of moisture stress on total phosphorus and total nitrogen content for the 3rd pair of laminae are illustrated in table II. Moisture stress has resulted in depressed accumulation of both nitrogen and phosphorus in these laminae.

The effects of moisture stress on the amount of nucleic acid in the 3rd pair of laminae is shown in figure 3. In the control plants, nucleic acid content of the 3rd pair of laminae increased continuously over the experimental period. In the plants subjected to water stress, however, nucleic acid content increased during the 1st 3-day period as moisture stress developed and then gradually decreased as the stress became more severe. Nucleic acid content per unit dry weight of leaf is similar in control and moisture stressed plants as is also shown in figure 3. There is therefore a close parallelism between nucleic acid content and dry weight of leaf. Treatment comparisons concerning total nucleic acid content are highly significant between the 2 treatments for the 4-day and 7-day harvests and between harvests at all occasions except for harvests at the 2- and 4-day periods in the moisture stressed series.

The experiment detailed in figures 2 and 3 and tables I and II shows therefore that moisture stress results in gradual cessation of nucleic acid accumulation in moisture stressed tomato leaves. The failure of the moisture stressed leaves to accumulate nucleic acid in a normal fashion might in principle be due either to failure of the stressed leaves to synthesize nucleic acid or to its excessive destruction. In order to distinguish between these two possibilities, experiments were carried out in which P^{32}-labeled phosphorus was supplied to control and to moisture stressed plants. These are essentially pulse experiments in which label is given for a period which is brief compared to the duration of the experiment. The labeled phosphate is then quickly diluted, presumably by phosphate taken up by the roots. After the plants were supplied with P^{32}-labeled phosphorus, incorporation into RNA occurred rapidly and reached a maximum level in about 3 hours. At this time the initial harvest of leaves was made. The fate of the label in the RNA was followed with time in the leaves of the remaining sets of plants. For these experiments plants were grown under controlled environmental conditions. Young plants were first grown in the Earhart Plant Research Laboratory until they had reached the age of 28 days and the 5th leaf was approximately 1.25 inches long. The plants were then removed to an artificially lighted (600 ft-c at the leaf surface) temperature controlled (25° ± 1° C) chamber which was also appropriately shielded for isotopic studies.

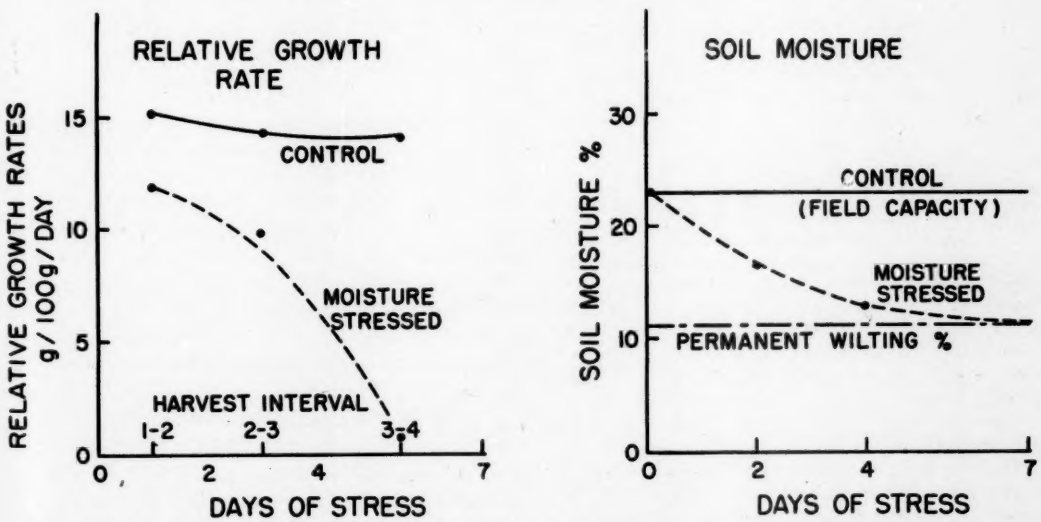


Fig. 2. Growth rates and soil moisture behavior for tomato plants during a single cycle of moisture stress under greenhouse conditions.

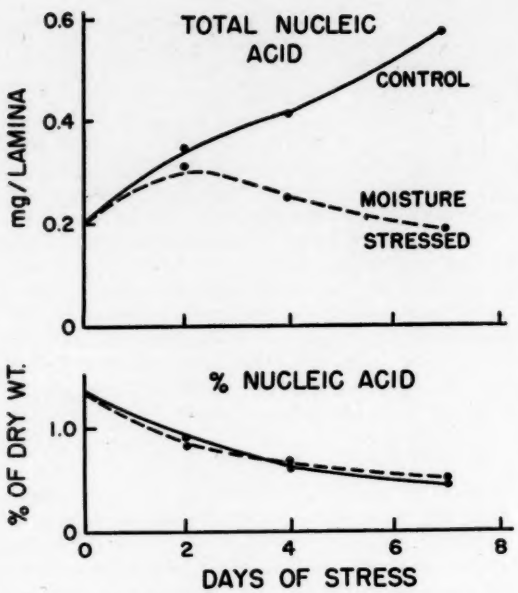


FIG. 3. Concentration and amount of nucleic acid in laminae of the 3rd pair of leaves of tomato plants during a single cycle of moisture stress under greenhouse conditions.

In the experiment of figure 4 the tops of all plants were immersed for 180 minutes in a solution containing radioactive phosphate, 0.5 microcurie per ml. The plants were then placed in the controlled condition chamber and moisture stress allowed to develop in one series. The laminae of leaf 5, which was at the stage of rapid expansion at the time treatment was commenced, were harvested for analysis at appropriate intervals. The data of figure 4 show that RNA continued to accumulate in the laminae of the control series plants, but failed to accumulate in the laminae of the moisture stressed plants.

TABLE I

DRY WEIGHTS OF CONTROL AND MOISTURE STRESSED PLANTS AND LEAVES* DURING DEVELOPMENT OF MOISTURE STRESS UNDER GREENHOUSE CONDITIONS

DAYS OF MOISTURE STRESS	DAYS FROM SOWING	HARVEST NO.	DRY WEIGHTS: G/PLANT			
			WHOLE PLANT		3RD PAIR LAMINAE	
			CONTROL	MOISTURE STRESSED	CONTROL	MOISTURE STRESSED
0	31	1	.80	...	.036	...
2	33	2	1.09	1.016	.083	.068
4	35	3	1.45	1.23	.140	.083
7	38	4	2.20	1.24	.2323	.075

* 3rd pair.

Figure 4 also presents data on the total and specific radioactivity of the RNA of the laminae in this experiment. In the control series specific activity of the RNA phosphorus decreases with time. Total activity of RNA phophorus remains approximately constant. The result indicates that in the control series phosphorus has been incorporated into RNA immediately after application of the isotopic material and that RNA synthesis has thereafter continued at the expense of the unlabeled phosphorus taken up by the roots from the nutrient solution. The labeled RNA, once made, is degraded but little over the time period of the experiment. In the wilted or moisture stressed plants, RNA content does not increase but remains constant with time. Specific activity of the RNA, however, decreases with time. Total radioactivity recovered in RNA also decreases with time, and after 5 days is only one half to two thirds of that initially present. This result indicates then that in the moisture stressed series, too, synthesis of RNA has continued at the expense of the unlabeled pool and that the labeled RNA made immediately after application of the P^{32}-labeled phosphate is being gradually destroyed. In this experiment all of the crude RNA hydrolysates were further purified by the methods indicated earlier. The conclusions which may be drawn from the measurements on the purified nucleotides are in agreement with those reached above on the basis of the determinations on the crude RNA hydrolysates and are therefore not presented.

TABLE II

TOTAL NITROGEN AND TOTAL PHOSPHORUS CONTENTS OF THE LAMINAE OF THE 3RD PAIR OF LEAVES DURING DEVELOPMENT OF MOISTURE STRESS UNDER GREENHOUSE CONDITIONS

DAYS OF MOISTURE STRESS	DAYS FROM SOWING	HARVEST NO.	NITROGEN, MG/PAIR LAMINAE		PHOSPHORUS, MG/PAIR LAMINAE	
			CONTROL	MOISTURE STRESSED	CONTROL	MOISTURE STRESSED
0	31	1	2.8	...	0.38	...
2	33	2	4.8	4.2	0.72	0.57
4	35	3	6.5	4.7	1.06	0.60
7	38	4	7.3	4.0	1.42	0.55

Table III also contains information on the amount and specific activity of the DNA of the laminae of this experiment. The amount of DNA in the laminae of the control plants increases steadily during the period of the experiment. It may be supposed therefore that chromosomal multiplication is continuing to occur.

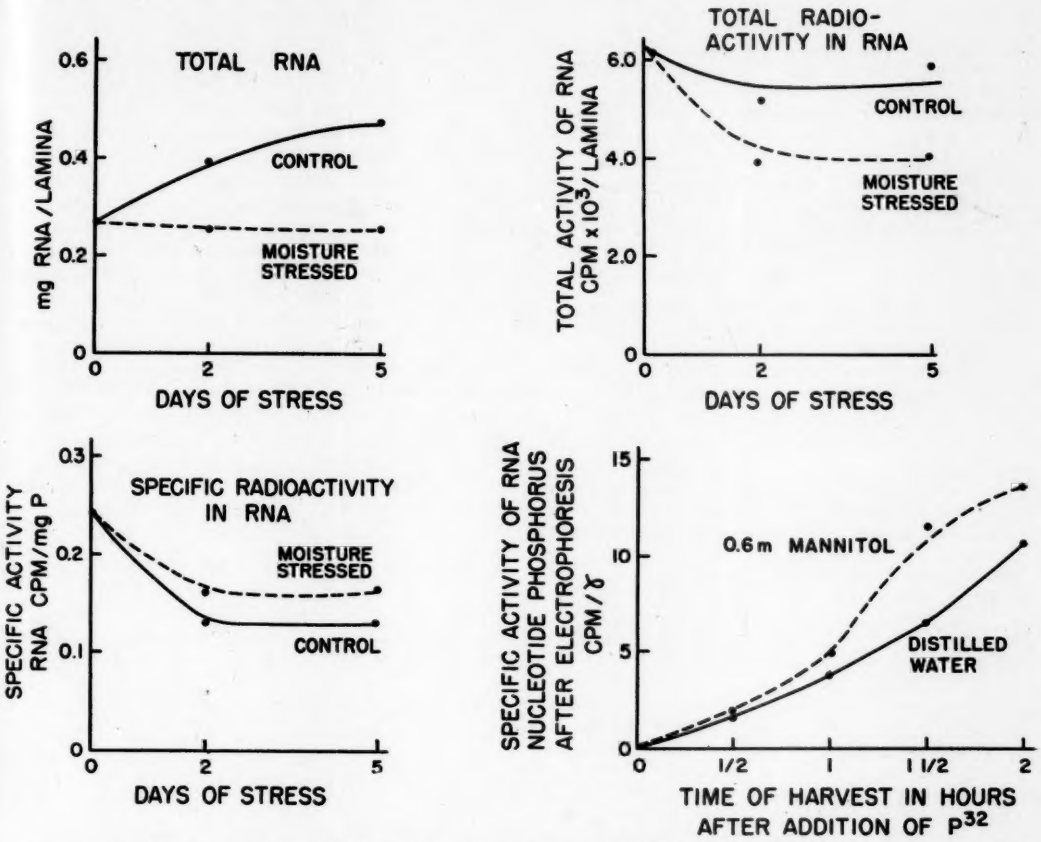


FIG. 4 (*top left and right and bottom left*). RNA content and P^{32} activity in RNA of tomato leaves during a single cycle of moisture stress. P^{32}-labeled phosphate incorporation into leaf RNA for 3 hours before the initial harvest.

FIG. 5 (*bottom right*). Specific activity of RNA of tomato leaves during incubation in P^{32}-labeled phosphate solution and in either water or 0.6 M mannitol.

TABLE III

EFFECT OF MOISTURE STRESS ON THE FATE OF RNA IN THE LAMINAE OF GROWING LEAVES (NO. 5) OF TOMATO PLANTS

	LAMINAE HARVEST AND TREATMENT:				
	INITIAL	AFTER 2 DAYS		AFTER 5 DAYS	
		CONTROL	MOISTURE STRESSED	CONTROL	MOISTURE STRESSED
RNA per lamina μg P	26	39	25	47	25
Specific activity of RNA, cpm/γ P*	238	130	157	126	160
DNA per lamina μg P	6.4	7.9	5.7	10.1	6.5
Specific activity of DNA, cpm/γ P	103	68	51	63	64

* P^{32}-labeled phosphate applied to leaves for a 3-hour period before the initial harvest and then withdrawn.

In the moisture stressed leaves, on the contrary, DNA content remains constant suggesting cessation of chromosomal multiplication.

As a further test of the hypothesis that moisture stressed plants continue, for a time at least, to synthesize RNA, a further type of experiment was done. Plants were allowed to develop moisture stress until they became wilted. The tops of stressed and control plants were then immersed in P^{32}-phosphate-containing solution (0.5 microcurie per milliliter). The plants were allowed to incubate for an additional 3 hours and leaves (the 4th lamina in this case) harvested for RNA determination. The results of such an experiment are given in table IV. RNA became labeled both in moisture stressed and in the control leaves and, in fact, the specific activity of the RNA of the moisture stressed leaves is approximately equal to that of the control leaves.

In another type of experiment, leaf 4 was detached from control plants and from plants suffering from acute water shortage. The petioles of these leaves

TABLE IV

RNA CONTENT AND RATE OF SYNTHESIS IN LAMINA 4 IN PLANTS SUBJECTED FOR 2 DAYS TO DEVELOPING MOISTURE STRESS

TREATMENT OF PLANTS	RNA PHOSPHORUS	SPECIFIC ACTIVITY* OF RNA PHOSPHORUS	WATER CONTENT OF LAMINAE
	μg	% dry weight	cpm/γ P	% dry weight
Control	66	.109	47	800
Moisture stressed	52	.115	41	760

* Plants dipped in P^{32}-solution after the 2 days of developing moisture stress. Incorporation allowed to continue for 3 hours.

TABLE V

RNA AND DNA PHOSPHORUS CONTENT AND ACTIVITY IN LAMINA 4 FOLLOWING RECOVERY FROM SEVERE MOISTURE STRESS FOR 5 HOURS IN P^{32}-LABELED PHOSPHATE SOLUTION

TREATMENT OF PLANTS	FRESH WEIGHT OF LAMINAE	RNA PHOSPHORUS PER LAMINA	SPECIFIC ACTIVITY OF RNA PHOSPHORUS	DNA PHOSPHORUS
	g	μg	(cpm/γ P)	μg
Control	.57	52	919	11.9
Moisture stressed	.51	37	816	9.9

were then placed in radioactive phosphate solution in the controlled light chamber so that phosphate was taken up through the petioles. After 5 hours of incubation the leaves were harvested and RNA determined in the usual way. The results of such an experiment are given in table V. The specific activity of the RNA of the control leaves is essentially equal to that of the initially wilted leaves.

EXPERIMENTS WITH OSMOTICALLY IMPOSED WATER STRESS: The above experiments indicate clearly that the leaves of moisture stressed plants possess the ability to incorporate phosphate into RNA although they do not possess the ability to carry on net accumulation of RNA. It might be objected, however, that the techniques used, immersion of the tops, or dipping of detached leaves into aqueous solutions of phosphate, always involve temporary relief of the water shortage to which the plants have been subjected earlier. It might therefore be possible that rapid synthesis of RNA occurs during this period of relief from moisture stress. It would clearly be of advantage to allow absorption of P^{32}-labeled phosphate into the tissue to proceed without relief of moisture stress. This has been attempted with osmotically imposed water stress.

In these experiments leaves were removed from tomato plants and equilibrated with solutions of varying osmotic concentration. Mannitol was used as the metabolically inert osmotically active solute. In all cases approximately half-expanded leaflets were used, in general of the 4th or 5th leaves. The plants used were approximately 28 days of age and replicate sets of 4 leaflets from each of 5 randomly selected, standard plants were used in each treatment of each experiment. Equilibration and subsequent incubation was carried on in the light in the controlled light chamber. After equilibration for 2.5 hours P^{32}-labeled phosphate was supplied to the solution in a final concentration of 0.5 microcurie per milliliter and at the desired osmolarity. Sets of leaves were harvested at intervals thereafter and RNA concentration and specific activity determined. The results of an experiment in which leaflets from leaf 4 were incubated in solutions of differing osmotic concentration are given in table VI. In this experiment the 2.5 hours of osmotic equilibration were followed by 1 hour of incubation in labeled phosphate. P^{32} is incorporated into RNA over the entire range of osmotic concentrations from 0 to 0.6 M, the latter 2 concentrations being those in which the leaf becomes flaccid. As is evident in table VI, specific activity of RNA is greater the higher the osmotic concentration to which the leaf is subject.

TABLE VI

INCORPORATION OF P^{32} INTO RNA OF LEAFLETS OF LEAF 4 AS AFFECTED BY EQUILIBRATION WITH VARYING CONCENTRATIONS OF MANNITOL

	EQUILIBRATION SOLUTION:*			
	DISTILLED WATER	0.2 M MANNITOL	0.4 M MANNITOL	0.6 M MANNITOL
RNA phosphorus per lamina: μg	62	60	62	60
Specific activity of RNA nucleotides after electrophoresis (cpm/γ nucleotide P)	4.2	4.2	7.0	7.7

* Equilibration for 2.5 hours followed by addition of P^{32}-labeled phosphate and incorporation for 1 further hour.

The time course of incorporation of labeled phosphate into RNA of leaflets of leaf 4 incubated in water or in 0.6 M mannitol is given in figure 5. Incorporation of P^{32} into RNA takes place initially at the same rate in the two series but the rate of incorporation gradually increases with time in the leaves incubated at the higher osmotic concentration.

No analytically detectable differences in total RNA content developed over the short incubation periods used in the experiments of table VI and figure 5. In order to assess the effects of high osmotic concentration on net RNA synthesis of excised leaves, a further experiment was carried out, in which leaflets were incubated for 4 days in a graded series of mannitol concentrations. The results of this experiment are given in table VII. There is net loss of

TABLE VII

EFFECTS OF OSMOTICALLY INDUCED MOISTURE STRESS ON THE RNA CONTENT OF LEAFLETS IN AN EXPERIMENT INVOLVING EXPOSURE FOR 4 DAYS TO A GRADED SERIES OF MANNITOL CONCENTRATIONS

	Equilibration solution			
Harvest	Distilled water	0.2 M mannitol	0.4 M mannitol	0.6 M mannitol
	μg RNA P/lamina			
Initial	65	...	...	...
After 4 days equilibration	56	61	49	35

RNA during incubation of the leaf tissue at all osmotic concentrations but such loss is greater the higher the osmotic concentration.

DISCUSSION

Under the influence of water stress net accumulation of RNA ceases in young tomato leaves. The changes in net accumulation of RNA closely parallel the changes in total dry weight of the leaves concerned. The fact that P^{32}-labeled phosphorus is rapidly incorporated into leaves of moisture stressed plants indicates that the block to net accumulation is not a block in ability to synthesize RNA. On the contrary, two kinds of evidence suggest that the block to net RNA synthesis under conditions of moisture stress consists in accelerated destruction of RNA. The first piece of evidence which bears on this point is the fact brought out in table III which concerns experiments in which a pulse of P^{32} is supplied to plants at the beginning of a moisture stress cycle. In the pulse experiment, P^{32} is rapidly incorporated into RNA. In control plants, the labeled RNA remains in the leaf although it is diluted by synthesis of further unlabeled RNA. In moisture stressed plants, on the contrary, labeled RNA is degraded, at least during the 1st harvest interval, although net content of RNA remains constant. The second piece of evidence which bears upon the matter is that obtained from leaves incubated on solutions of varying osmotic concentration. It is apparent that sufficiently high osmotic stress causes leaves to lose RNA more rapidly than they would otherwise do although they retain the ability to incorporate labeled phosphate into RNA and hence presumably to synthesize RNA.

SUMMARY

1. It is already known that moisture stress inhibits not only growth and dry weight accumulation of young tomato leaves but also the accumulation by such leaves of nitrogen and phosphorus. The present work has shown that moisture stress also suppresses net increases in the RNA of such leaves.

2. The leaves of moisture stressed tomato plants possess the ability to incorporate P^{32}-labeled phosphate in RNA even though they do not exhibit any net synthesis of the material.

3. It is concluded that in the leaves of moisture stressed tomato plants, rate of destruction of RNA may be increased above the rate characteristic of control, non moisture stressed leaves. This conclusion is further supported by the experimental fact that osmotically induced moisture stress increases rate of RNA loss in excised tomato leaves.

We wish to acknowledge the advice and counsel of Dr. Kenneth Glasziou, Dr. Clifford Sato, Dr. Paul O. P. Ts'o and Dr. George G. Laties.

LITERATURE CITED

1. GATES, C. T. The response of the young tomato plant to a brief period of water shortage. I. The whole plant and its principal parts. Australian Journ. Biol. Sci. 8: 196-214. 1955.
2. GATES, C. T. The response of the young tomato plant to a brief period of water shortage. II. The individual leaves. Australian Journ. Biol. Sci. 8: 215–230. 1955.
3. GATES, C. T. The response of the young tomato plant to a brief period of water shortage. III. Drifts in nitrogen and phosphorus. Australian Journ. Biol. Sci. 10: 125–146. 1957.
4. GERRITZ, H. W. Report on the determination of P_2O_5 in jams, jellies and other fruit products. Journ. Assoc. Offic. Agr. Chem. 23: 321–334. 1940.
5. OGUR, M. and ROSEN, G. The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25: 262–276. 1950.
6. SCHMIDT, G. and THANNHAUSER, S. J. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. Journ. Biol. Chem. 161: 83–89. 1945.
7. TAYLOR, J. K. and HOOPER, P. D. A soil survey of the horticultural soils in the Murrumbidgee irrigation areas, N.S.W. Journ. Council Sci. Ind. Research (Australia) Bull. 118. 1938.
8. WYATT, G. R. The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. Journ. 48: 584–590. 1951.

CHLOROPLAST ISOLATION IN NONAQUEOUS MEDIA[1,2]

C. RALPH STOCKING

DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

An understanding of the physiological role of a particular enzyme in cell metabolism rests in part on a knowledge of the exact intracellular site of action of that enzyme. Both histochemical tests (22) and the differential centrifugation of cell free homogenates (10) have yielded valuable information concerning the intracellular location of some enzymes in plants. Unfortunately, reliable histochemical tests are available for only a few enzymes, and the possibility of artifacts arising as a result of isolation procedures often negates the conclusions obtained by means of the differential centrifugation technique. Of particular importance in this connection are the leaching of enzymes from morphological units and the possibility of adsorption of soluble enzymes by such structures during their isolation in aqueous media. A further difficulty that may be encountered is the inactivation of enzymes resulting from a mixing of the cell contents during grinding and separation in aqueous media (7).

The extensive use, in physiological and biochemical studies, of chloroplast isolation in aqueous media makes it imperative that we increase our knowledge concerning the effects of these techniques on the loss of large molecular weight substances such as enzymes and cofactors from the plastids during their isolation. This point has been emphasized by McClendon (14) in his studies on the effect of polyethylene glycol upon the loss of phycocyanin from red algal chloroplasts. The necessity of adding a "cytoplasmic factor" back to isolated chloroplasts to attain maximum activity is indicative of the problem. A water soluble chloroplast extract appears to function in phosphorylation by reducing triphosphopyridine nucleotide in the light (4, 17). Similarly Avron and Jagendorf (5) found an extractable chloroplast factor associated with photosynthetic phosphorylation.

Certain water soluble enzymes, which because of their role in photosynthesis or in starch synthesis would be expected to be found chiefly in chloroplasts, have been reported to be present primarily in the supernatant from chloroplasts isolated in aqueous media. This is true of aldolase (21) and phosphorylase (15, 20).

In order to overcome some of the difficulties involved in the isolation of nuclei in aqueous media, Behrens (6) developed a procedure whereby nuclei could be isolated from lyophilized heart muscle without the use of aqueous solutions. This method has been modified recently and used in the study of nuclei isolated from animal tissue (1, 8, 9, 12). However, with the exception of the isolation of wheat germ nuclei (19), the nonaqueous technique has not been reported to have been employed on plant material.

The present paper describes a modification of Behrens' technique that has been used in the preparation of chloroplasts.

MATERIALS AND METHODS

CHLOROPLAST ISOLATION: Unless otherwise noted, leaf material was harvested from greenhouse grown tobacco plants (*Nicotiana rustica* L.) that had been in continuous darkness for 36 to 48 hours prior to harvest. After the leaves were cut from the plants and the midvein and major laterals were removed with a razor, the leaf strips were dropped immediately into a lyophilization flask immersed in a freezing mixture of acetone and dry ice. While frozen, the brittle leaves were broken into fragments ranging up to 1 cm^2 in area. It was found that extreme care should be exercised to prevent thawing of the leaves during the lyophilization. The flask holding the frozen leaves was kept at approximately $-10°$ C in a salt-ice mixture during the lyophilization. The water condensation trap was cooled with a dry ice-acetone mixture. After complete drying, the samples were stored under vacuum at $-5°$ C until used. All steps in the isolation were carried out at approximately 0° C. The solvents were precooled and a refrigerated centrifuge was used.

The procedure for the separation is outlined in figure 1. Several methods of grinding were tested, and it was found that small samples could be most efficiently and rapidly ground with a minimum of plastid rupture if a hand model tissue grinder was used. Generally, two 0.1-g samples of the lyophilized leaf were ground in a Ten Broeck hand model tissue grinder with about 20 ml of an ice cold hexane/carbon tetrachloride mixture having a density of 1.32. In some experiments the first grinding medium had a density of 1.28 or 1.30 rather than 1.32. The density of chloroplasts varies with previous history of the plant as well as with leaf age (11). Approximately two to four minutes of gentle grinding was sufficient to reduce the sample to a uniform appearing homogenate. The ground samples were combined, filtered through a glasswool cheesecloth pad, diluted to known volume with the homogenization liquid, and aliquots were removed for chlorophyll, nitrogen, and enzyme determinations. The remainder of the homogenate

[1] Received July 14, 1958.

[2] This work was carried out in part when the author was a Merck Post-doctoral Fellow in Biochemistry, University of Wisconsin.

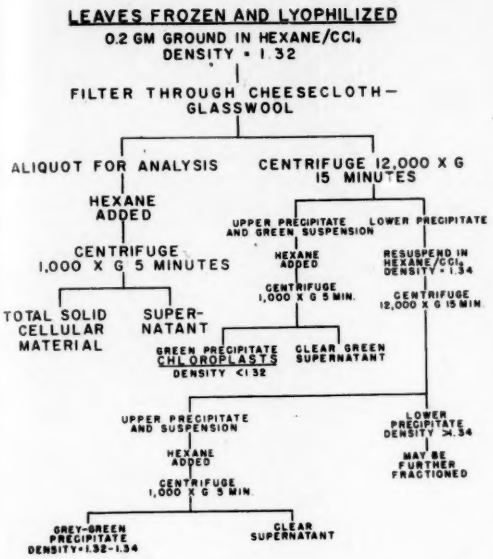


FIG. 1. Procedure for the isolation of chloroplasts in nonaqueous media.

was centrifuged for 15 minutes at 12,000 × G in polyethylene tubes.

Centrifugation resulted in the deposition of a green pellet on the wall of the centrifuge tube near the surface of the liquid. This green pellet, together with the green liquid, was separated from the more dense material that had been deposited at the bottom of the tube. An equal volume of hexane was mixed with the light fraction. This reduced the density of the liquid below that of the suspended particulate matter, and the latter was subsequently deposited as a pellet when the mixture was centrifuged for five minutes at 1000 × G. The pellet thus obtained contained primarily chloroplasts which were either dried under vacuum or washed by resuspending them in a solution of hexane and carbon tetrachloride having a density of 1.32. Washing was followed by recentrifugation and vacuum drying.

The material, more dense than 1.32, that was deposited at the bottom of the tube after the first centrifugation of the homogenate was resuspended in a hexane and carbon tetrachloride mixture having a density of 1.34, and the separation was continued as outlined in figure 1.

BIOCHEMICAL TESTS: Aliquots of each fraction were extracted with 80 % acetone and optical density readings were taken at 652 mμ. Chlorophyll concentrations were calculated from these data by Arnon's method (2).

Total nitrogen and trichloroacetic acid insoluble nitrogen were determined by a micro-kjeldahl method in which mercuric sulfate was used as a catalyst.

Enzyme assays were carried out within the range in which the rates of the reaction were proportional to the concentration of the enzyme.

Phosphorylase analyses were made by the method previously described in which the inorganic phosphorus liberated from glucose-1-phosphate was measured (20). In this method phosphatase activity is inhibited by 0.01 M sodium fluoride.

Aldolase was determined by following the appearance of alkali-labile phosphate under the reaction conditions described by Roodyn (16). The reaction mixture contained 0.056 M hydrazine sulfate, 0.04 M TRIS buffer pH 8.6, and 0.005 M sodium hexose diphosphate. The reaction, carried out at 37° C, was stopped after 15 minutes by the addition of iced trichloroacetic acid to give a final concentration of 4.5 %. The cold tubes were centrifuged and the inorganic phosphate and alkali-labile phosphate of the supernatant was determined.

The purified plastids isolated from tobacco leaves contain prominent grana-like areas and are dicoid in shape. However, many appear to have an indentation in the center, which causes them to take on a shape somewhat similar to that of a tennis ball pushed in on one side (fig 3). Plastids of this shape have been observed frequently in sections prepared from leaves of plants kept in the dark for two or three days.

As would be expected, an appreciable portion of the total pigment content of the leaf is removed during the grinding and isolation in hexane/carbon tetrachloride. However, the chlorophyll removal occurred primarily during the initial grinding period and subsequent washes of the chloroplasts with the cold solvents removed only a relatively small amount of the remaining chlorophyll. Presumably much of the chlorophyll initially extracted came from chloroplasts broken during the grinding.

Although the fraction more dense than 1.32 is a mixture of materials and not entirely free of trapped or heavy chloroplasts, some of the components can be partially separated. Thus, nuclei are most abundant in a fraction having a density between 1.34 and 1.36, while the majority of cell wall fragments are more dense than 1.44. Figure 5 is a photomicrograph of a composite fraction more dense than 1.40.

FATE OF WATER SOLUBLE VACUOLAR COMPONENTS: In order to test the efficiency with which the nonaqueous method could separate chloroplasts from water soluble vacuolar constituents, a comparison was made between the anthocyanin and the chlorophyll distribution in the various fractions isolated from Irisene leaves. In the original leaf material, anthocyanin completely masks the chlorophyll. It can be seen from figure 6 that the most purified chloroplasts were essentially free of anthocyanin, while the fractions with greatest density contain the major amount of this vacuolar pigment.

PHOSPHORYLASE: A comparison was made of the intracellular distribution of phosphorylase determined by several methods of isolating chloroplasts in aqueous media with the intracellular distribution determined by the nonaqueous technique. It is apparent from the

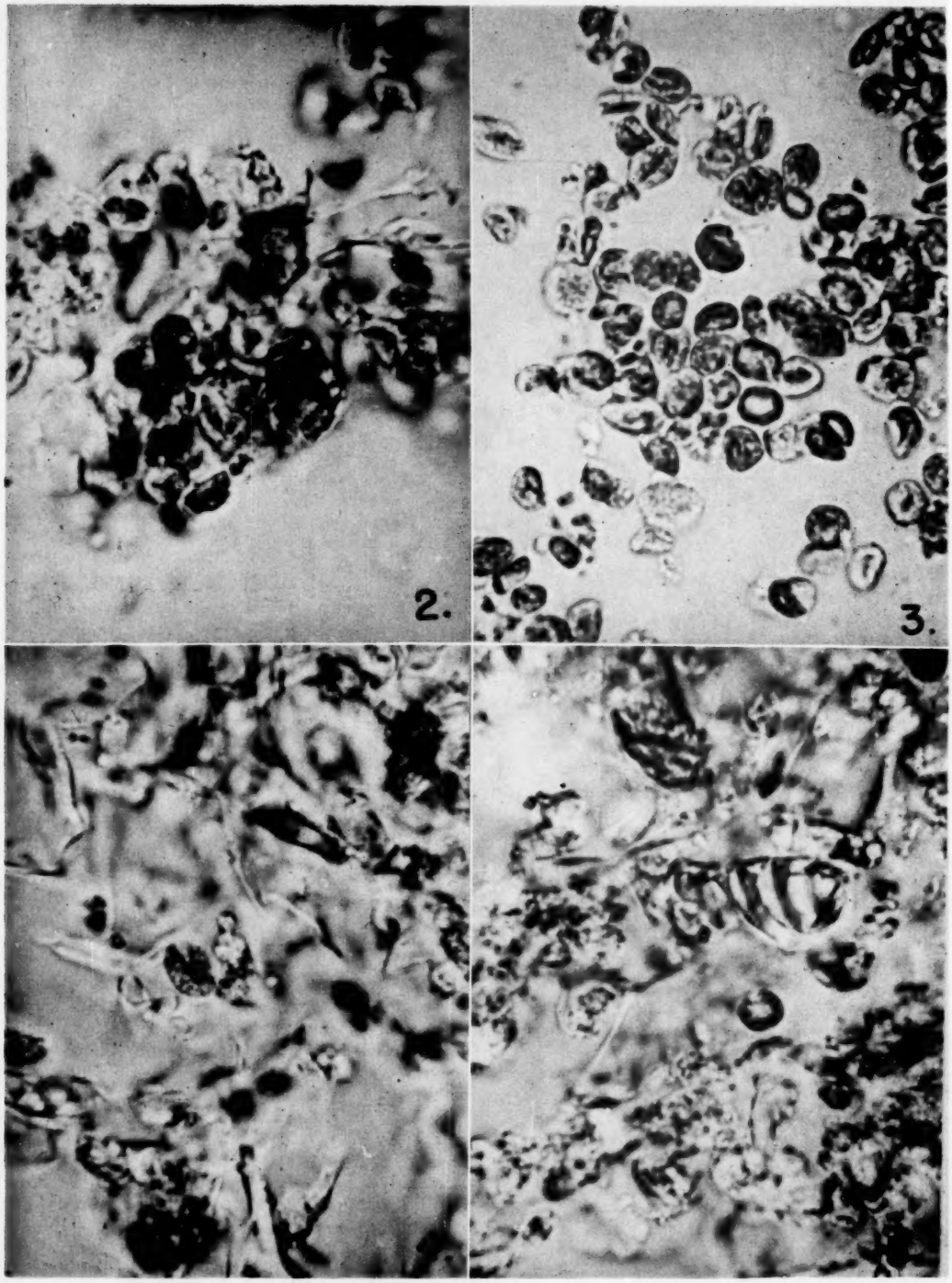


FIG. 2. Lyophilized and ground leaf material before chloroplast isolation. ×2000.
FIG. 3. Chloroplasts isolated in nonaqueous media. ×2000. Density less than 1.32.
FIG. 4. Fraction of density 1.32 to 1.40. ×2000.
FIG. 5. Fraction of density greater than 1.40. ×2000.

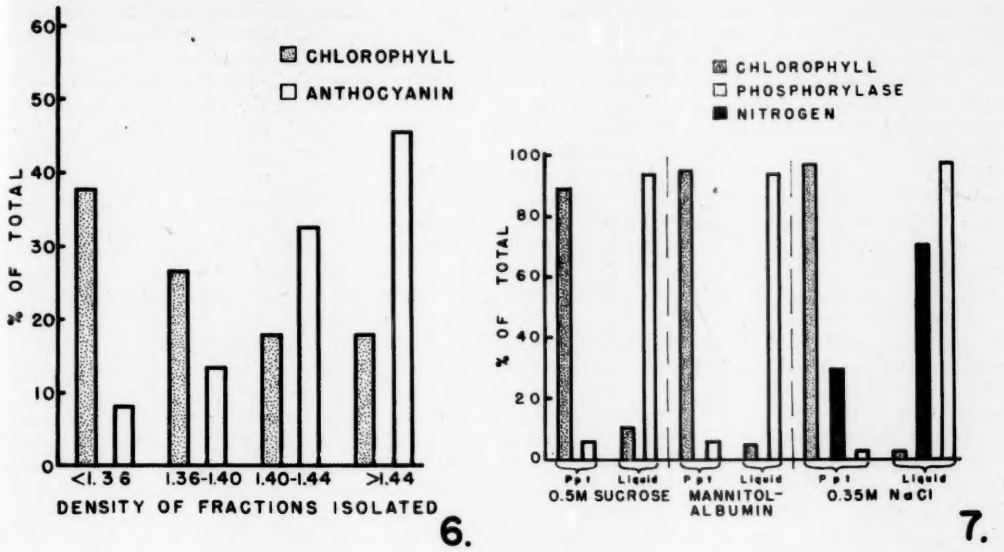


FIG. 6. Anthocyanin and chlorophyll content of fractions isolated from Irisene leaves by the nonaqeous method.

FIG. 7. A comparison of the intracellular distribution of phosphorylase in tobacco leaves as determined by 3 methods of isolation of chloroplasts in aqueous media.

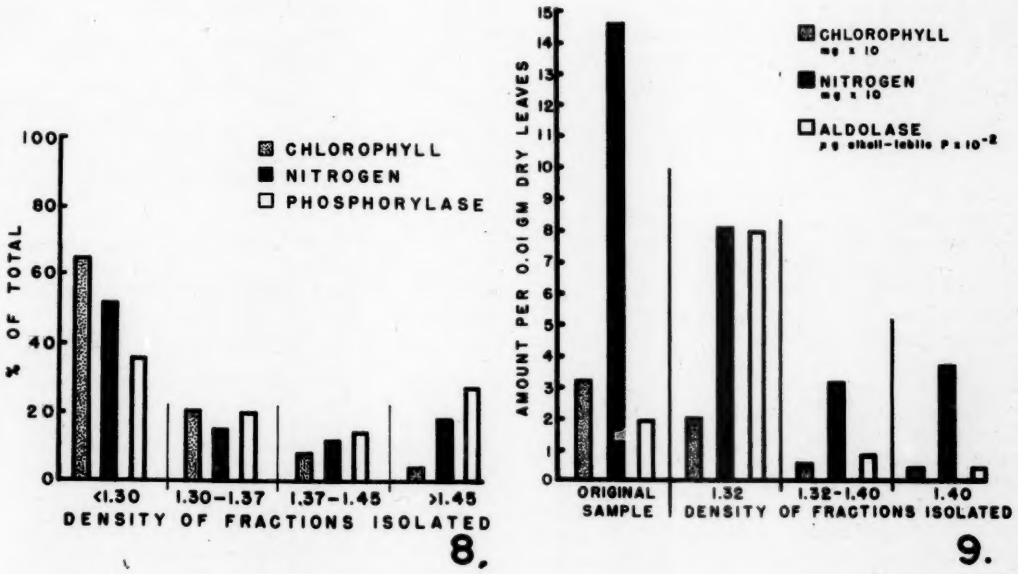


FIG. 8. Phosphorylase, chlorophyll, and nitrogen content of fractions isolated from tobacco leaves by the nonaqueous method.

FIG. 9. Aldolase, chlorophyll, and nitrogen content of fractions isolated from tobacco leaves by the nonaqueous method.

data reported in figure 7 that over 90 % of the phosphorylase is located in the supernatant when chloroplasts are isolated in a variety of aqueous media. This is in agreement with results already published (15, 18, 20). On the other hand, the most pure fraction of chloroplasts (density < 1.32) isolated in nonaqueous media contained 65 % of the recovered chlorophyll and 35 % of the recovered phosphorylase (fig 8). If we ignore possible unequal loss of enzyme and chlorophyll from ruptured plastids and accept these figures as indicating that 65 % of the chloroplasts containing 35 % of the total phosphorylase are recovered in the < 1.32 density fraction, then at least 54 % of the cell phosphorylase is associated with the total chloroplasts in the cell.

ALDOLASE: Twefik and Stumpf (21) reported that, when chloroplasts and chloroplast fragments were isolated in 0.4 M sucrose, the supernatant contained essentially all of the leaf aldolase. However, aldolase has been implicated as one of the enzymes involved in CO_2 fixation during photosynthesis (23), and Arnon (3) has reported CO_2 fixation in chloroplasts isolated in salt solutions. Consequently, one should expect aldolase to be present in the chloroplasts of living cells. In order to study this point, the aldolase distribution in fractions obtained from leaves by the nonaqueous technique was investigated.

A high aldolase activity was observed in the isolated chloroplasts even after washing them twice with a hexane/carbon tetrachloride mixture. A further interesting aspect of this experiment is that the chloroplast fraction isolated from tobacco leaves by the nonaqueous method showed several times the aldolase activity of the total unfractionated lyophilized leaf material (fig 9). Either the cytoplasmic fraction has an inhibitory effect on the aldolase activity or aldolase in an active form was liberated during the isolation. The significance of this lower activity of the entire homogenized sample is under investigation.

DISCUSSION

Lynch and French (13) reported that petroleum ether extracted chloroplasts are inactive in the Hill reaction. Similarly, observations so far have failed to demonstrate the presence of photochemical activity (Hill reaction) in chloroplasts isolated by the nonaqueous technique. In contrast to Lynch and French's results, no activity was observed even when the lipids dissolved in the isolation medium were added back to the chloroplasts. However, attempts to measure the Hill reaction were complicated by the presence of an active reductant (probably ascorbic acid) in the plastids isolated by this method.

Although the nonaqueous isolation method suffers from the obvious difficulties involved in the use of lyophilic solvents for the isolation of a highly lipid structure, and causes the inactivation of some enzymes (8), the results reported here indicate that this technique is useful in studying the intracellular location of certain enzymes. A combination of this procedure, together with the more common aqueous methods, should enhance our understanding of plastid composition. One difficulty with both methods, but perhaps more inherent in the nonaqueous technique, is the difficulty of removing the last fragments of hyaloplasm from the surface of the chloroplasts. Furthermore, an unambiguous test for the completeness of removal of such contaminants is difficult. Although the isolated chloroplasts can be washed with the isolation medium and visible contaminants essentially removed, more or less protein intimately associated with the plastid surface undoubtedly remains. A further difficulty encountered both with the nonaqueous method, as well as with the more familiar sucrose and salt methods of isolating chloroplasts, is the determination of the fate of the mitochondria.

However, if the possible presence of such contaminating material is taken into consideration, the ease with which enzymes such as aldolase and phosphorylase can be removed from chloroplasts during plastid isolation in sugar or salt solutions is emphasized here. Although the presence of a high aldolase activity in the chloroplasts has been shown, no estimate can be made of the fraction that this represents of the total leaf aldolase. Such a comparison will only be possible when the inhibitory action of total cell contents on aldolase activity is more clearly understood.

SUMMARY

A technique is described whereby chloroplasts can be isolated from lyophilized leaves without the use of an aqueous medium. Such chloroplasts do not contact liquid water at any time during their isolation. Consequently water soluble enzymes are not leached from them. Chloroplasts so isolated have a high aldolase activity and are associated with at least 54 % of the cell phosphorylase. The nonaqueous method of preparing chloroplasts, while suffering from difficulties associated with the use of lipid solvents, promises to be useful as a supplement to the aqueous methods in the study of the intracellular distribution of enzymes in leaves.

The author wishes to thank Dr. R. H. Burris for valuable discussion and advice and for the use of his laboratory, in which the initial phase of this work was carried out.

LITERATURE CITED

1. ALFREY, V., STERN, H., MIRSKY, A. E. and SAETREN, H. The isolation of cell nuclei in non-aqueous media. Jour. Gen. Physiol. 35: 529–578. 1952.
2. ARNON, D. I. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15. 1949.
3. ARNON, D. I., ALLEN, M. B. and WHATLEY, F. R. Photosynthesis by isolated chloroplasts. IV. General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta 20: 449–461. 1956.
4. ARNON, D. I., WHATLEY, F. R. and ALLEN, M. B. Triphosphopyridine nucleotide as a catalyst of photosynthetic phosphorylation. Nature 180: 182–185. 1957.

5. AVRON, M. and JAGENDORF, A. T. An extractable factor in photosynthetic phosphorylation. Nature 179: 428–429. 1957.
6. BEHRENS, M. Untersuchungen an isolierten Zell- und Gewebsbestandteilen. I. Mitteilung: Isolierung von Zellkernen des Kalbsherzmuskels. Zeits. physiol. Chem. 209: 59–74. 1932.
7. CLENDENNING, K. A. Biochemistry of chloroplasts in relation to the Hill reaction. Ann. Rev. Plant Physiol. 8: 137–152. 1957.
8. DOUNCE, A. L. Cytochemical foundations of enzyme chemistry. In: The Enzymes. J. B. Sumner and K. Myrbäck, eds. Vol. 1, part 1. Pp. 187–266. 1950.
9. DOUNCE, A. L., TISHKOFF, G. H., BARNETT, S. R. and FREER, R. M. Free amino acids and nucleic acid content of cell nuclei isolated by a modification of Behrens' technique. Jour. Gen. Physiol. 33: 629–642. 1950.
10. GODDARD, D. R. and STAFFORD, HELEN A. Localization of enzymes in the cells of higher plants. Ann. Rev. Plant Physiol. 5: 115–132. 1954.
11. JAGENDORF, A. T. Purification of chloroplasts by a density technique. Plant Physiol. 30: 138–143. 1955.
12. KAY, E. R. M., SMELLIE, R. M. S., HUMPHREY, G. E. and DAVIDSON, J. N. A comparison of cell nuclei isolated from rabbit tissue by aqueous and non-aqueous procedures. Biochem. Jour. 62: 160–168. 1956.
13. LYNCH, VICTORIA H. and FRENCH, C. S. β-Carotene, an active component of chloroplasts. Arch. Biochem. Biophys. 70: 382–391. 1957.
14. MCCLENDON, J. H. The physical environment of chloroplasts as related to their morphology and activity in vitro. Plant Physiol. 29: 448–458. 1954.
15. PAECH, K. and KRECH, E. Über die Starkebildung in den Plastiden. Planta 41: 391–395. 1953.
16. ROODYN, D. B. The enzymic properties of rat-liver nuclei. I. Estimation of the extent to which contaminant material contributes to the activity observed in nuclear fractions. Biochem. Jour. 64: 361–368. 1956.
17. SAN PIETRO, A. and LONG, HELGA M. Accumulation of reduced pyridine nucleotides by illuminated grana. Science 124: 118. 1957.
18. STAFFORD, HELEN A. Intracellular localization of enzymes in pea seedlings. Physiol. Plantarum 4: 696–741. 1951.
19. STERN, H. and MIRSKY, A. E. The isolation of wheat germ nuclei and some aspects of their glycolytic metabolism. Jour. Gen. Physiol. 36: 181–200. 1952.
20. STOCKING, C. R. The intracellular location of phosphorylase in leaves. Amer. Jour. Bot. 39: 283–287. 1952.
21. TWEFIK, S. and STUMPF, P. K. Carbohydrate metabolism in higher plants. II. The distribution of aldolase in plants. Amer. Jour. Bot. 36: 567–571. 1949.
22. VAN FLEET, D. S. Histochemical localization of enzymes in vascular plants. Bot. Rev. 18: 354–389. 1952.
23. VISHNIAC, W. Biochemical aspects of photosynthesis. Ann. Rev. Plant Physiol. 6: 115–134. 1955.

EFFECT OF 2,3,6-TRICHLOROBENZOIC ACID AND 2,6-DICHLOROBENZOIC ACID ON THE GEOTROPIC AND PHOTOTROPIC RESPONSES OF SEEDLINGS OF VARIOUS SPECIES[1,2,3]

LEO C. VANDER BEEK[4]

DEPARTMENT OF BOTANY, UNIVERSITY OF MICHIGAN, ANN ARBOR, MICHIGAN

Bentley (1), using the straight-growth Avena method as a criterion of auxin activity, showed that 2,3,6-trichlorobenzoic acid and 2,3,6-trichlorobenzaldehyde had high physiological activity. Zimmerman and Hitchcock (10) substantiated the auxin-like activity of 2,3,6-trichlorobenzoic acid and 2,3,6-trichlorobenzaldehyde reported by Bentley. Veldstra (8) and Thimann (6) reported the high activity of 2,3,6-trichlorobenzoic acid in the split-pea test. Wardlaw (9) observed that 2,3,6-trichlorobenzoic acid suppressed twining and leaf formation but increased root formation. Jones, Metcalfe and Sexton (2), in the course of their investigations, observed that 2,3,6-trichlorobenzoic acid abolished the normal geotropic and phototropic responses in ryegrass.

As a result of these investigations attention was focused on the halogenated benzoic acids. Of particular interest were the chlorinated benzoic acids. In 2,3,6-trichlorobenzoic acid was found a material which was unquestionably auxin-like, but which fails to fulfill the structural requirements of Koepfli et al (3), Veldstra (7) or Muir, Hansch and Gallup (5).

In this laboratory it was observed that 2,3,6-trichlorobenzoic acid caused a disturbance in the normal upright shoot growth of cucumber, barley, oats and other seedlings grown either in the dark or in the light. Few other compounds having auxin activity in some degree repress the geotropic and phototropic responses of roots and shoots. This clearly distinctive and unusual property of 2,3,6-trichlorobenzoic acid, therefore, seemed worthy of further attention.

[1] Received revised manuscript July 24, 1958.

[2] Paper no. 14 from the Plant Nutrition Laboratory. This work was supported by funds of the Ford Agricultural Plant Nutrition Project.

[3] From the Ph.D. thesis of Leo C. Vander Beek, University of Michigan, 1956.

[4] Present address: Western Michigan University, Kalamazoo, Michigan.

Materials and Methods

Shoots of barley seedlings treated with 2,3,6-trichlorobenzoic acid and grown in the dark do not then curve upward in the normal, negatively geotropic position. This failure appears to depend upon the level of treatment.

The method described here was devised to test the suspected quantitative relationship between level of treatment and amount of repression of the geotropic response.

Oats (*Avena sativa* L. var. Clinton) were selected for detailed studies. The long seeds are easier to handle than those of barley in the tests described below. Seeds were from certified seed stocks.

The Heyden Chemical Company, New York, kindly supplied 2,3,6-trichlorobenzoic acid (HC-1281, Lot 870-86) and other chlorinated benzoic acids.

The criterion in the test is the effectiveness with which the test solution decreases the angle formed by a line drawn through the long axis of the oat seed and another drawn parallel to the apical half of the oat shoot.

Hulled oats were placed in a small muslin bag and aerated in distilled water at 25° C for 24 hours. Then the seeds were inserted with embryo out at the edge of a cube of cellulose sponge. Each cube held one seed.

Sponges were prepared by boiling to remove any water-soluble material. Small cubes measuring approximately 1 cm on a side were cut from dried sponges. A small hole was drilled in one face of each cube close to an edge.

For a humid test chamber a glass refrigerator dish was used. The cube with seed was carefully placed in the inverted cover of the dish containing 125 ml of the test solution or distilled water. Twenty cubes were placed in each lid in four rows of five cubes each. Such spacing allows sufficient freedom to the growing coleoptile even when highly repressive concentrations of 2,3,6-trichlorobenzoic acid are tested. The inverted bottom of the refrigerator dish was then used as the top of the test chamber. All chambers were maintained in red light to prevent elongation of the mesocotyl at 25° C until the seedlings were 96 hours old.

At the end of 96 hours and still under red light the seedlings in one chamber were carefully removed from their cubes and placed in a row on a piece of glass in such a way that seed and shoot were in the plane of the glass. Any seedlings that had fallen from their cubes were discarded. A strip of Scotch brand tape was placed gently over the seedlings to hold them in place. The glass was placed on a piece of wood of similar size and a sheet of Kodabromide paper was inserted between the glass and the wood. The ensemble was placed vertically in a grooved block and a shadowgraph was made in a shadowgraph box, (Leopold, 4). Essential features of the procedure are illustrated in figures 1 and 2.

After development and drying, lines were drawn on the shadowgraph through the long axis of the seed and parallel to the apical half of the coleoptile. The included angle was measured with a protractor. The average of 60 to 80 such measurements was considered the repression elicited by the test solution.

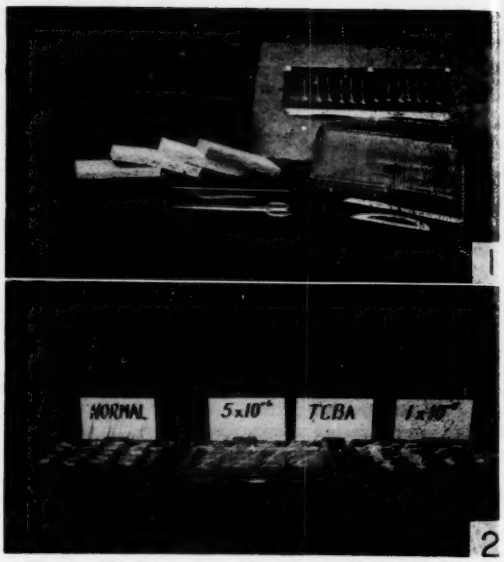


Fig. 1. Equipment employed in the oat repression test. On the left oat seedlings are shown taped to a small sheet of glass prior to being shadowgraphed. A developed shadowgraph is shown on the right.

Fig. 2. Oat seedlings prior to being shadowgraphed in the oat repression test. In the test, seedlings grow from cellulose sponge cubes placed in 2,3,6-trichlorobenzoic acid (TCBA).

A qualitative method was used to determine the effect of compounds upon the phototropic response of various species. The criterion of this test is the effectiveness of the compound in abolishing the phototropic response of shoots, grown in an aqueous solution of the compound, when placed in unilateral light.

To test such species as barley, oats, corn and cucumber, 25-mesh Saran screen was stretched across plastic containers. After 24 hours of aeration seeds were placed on the screen. The test solution barely touched the screen; the seeds were kept moist by covering them with moistened filter paper. Further aeration was not necessary as the test does not depend upon extensive root systems. Containers were maintained at 25° C in the dark until the seedlings were about 2 inches in height. The containers were then placed in unilateral light. After 24 hours, comparisons of untreated controls with treated seedlings were made.

Species having tiny seeds cannot conveniently be tested on the relatively large 25-mesh Saran screen. These were placed on filter paper in plastic containers. After the seedlings were well established, the containers with seedlings were placed in unilateral light.

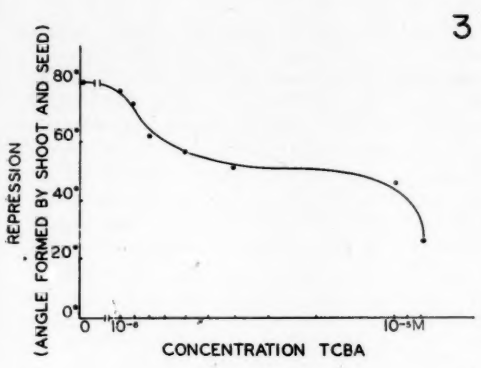
Results and Discussion

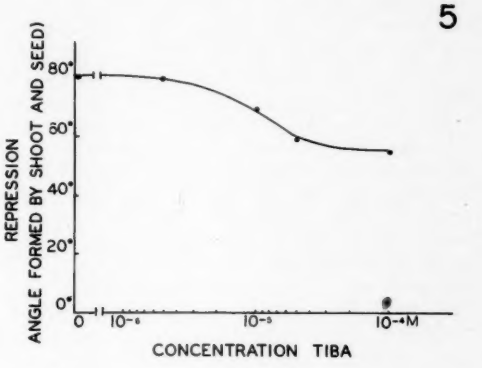
The results obtained in the oat repression test with various levels of 2,3,6-trichlorobenzoic acid are shown in figure 3. At 96 hours the upward curvature of the shoot is not complete; the included angle in untreated controls is approximately 80°.

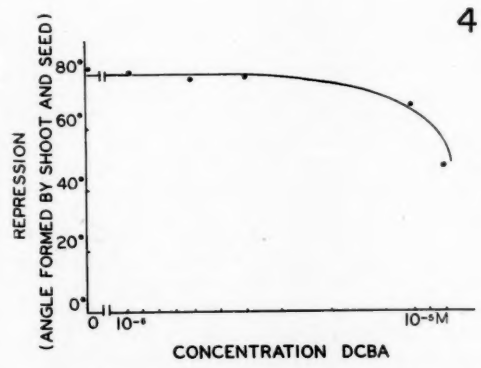
It is quite apparent in figure 3 that a relationship exists between extent of repression and level of treatment. At 5×10^{-5} M, the angle approaches 0° and shoots, as they emerge, come in contact with the face of the sponge. Many shoots because of this "push themselves" out of the hole in the sponge at immediately higher levels making it difficult to obtain consistent results.

It has been observed in this laboratory that 2,6-dichlorobenzoic acid caused shoots of barley, timothy, clover and tobacco to exhibit a lack of uniformity similar to that elicited by 2,3,6-trichlorobenzoic acid. To determine if there exists, as in 2,3,6-trichlorobenzoic acid, a relationship to treatment level, 2,6-dichlorobenzoic acid was also tested in the oat repression test. The results are shown in figure 4. Although the results show that repression of the geotropic response with 2,6-dichlorobenzoic acid also depends upon concentration, comparison of figures 3 and 4 shows that 2,6-dichlorobenzoic acid is much less active than 2,3,6-trichlorobenzoic acid.

Two compounds prominent in the literature of growth regulators are 2,4-dichlorophenoxyacetic acid and 2,3,5-triiodobenzoic acid. Jones et al (2) reported that 2,3,5-triiodobenzoic acid inhibited the geotropic response of rape and ryegrass roots when such seedlings were grown on agar containing the compound.







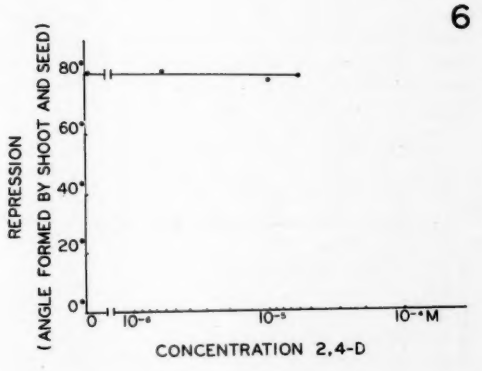


Fig. 3. The effect of various levels of 2,3,6-trichlorobenzoic acid (TCBA) upon the geotropic response of Avena shoots grown for 96 hours at 25° C.

Fig. 4. The effect of various levels of 2,6-dichlorobenzoic acid (DCBA) upon the geotropic response of Avena shoots grown for 96 hours at 25° C.

Fig. 5. The effect of various concentrations of 2,3,5-triiodobenzoic acid (TIBA) upon the geotropic response of Avena shoots grown for 96 hours at 25° C.

Fig. 6. The effect of various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) upon the geotropic response of Avena shoots grown for 96 hours at 25° C.

To determine the effect of the compound on the geotropic response of shoots, it was tested in the oat repression test (fig 5). The data indicated that 2,3,5-triiodobenzoic acid does repress the geotropic response in oat shoots, but it is clearly much less active than 2,3,6-trichlorobenzoic acid. The repression obtained at 1×10^{-4} M (56°) is produced by 2,3,6-trichlorobenzoic acid at 5×10^{-6} M.

Results obtained with 2,4-dichlorophenoxyacetic acid in the oat repression test are given in figure 6. Even high levels fail to produce appreciable repression of the geotropic response.

In culture experiments with barley or oats the shoots emerge at 48 hours or shortly thereafter. At the time of emergence no differences are observable between the shoots of barley or oats treated with 2,3,6-trichlorobenzoic acid and those of untreated controls. As roots are already in contact with the test solution at shoot emergence, it appears that contact with 2,3,6-trichlorobenzoic acid, at least during the first 48 hours does not cause an abnormal geotropic response. That this is indeed the case during the first 24 hours was shown by aerating oats for 24 hours at 25° C in 1×10^{-5} M 2,3,6-trichlorobenzoic acid, in which concentration was found a marked decrease of the normal geotropic response of oat seedlings after 96 hours (fig 3). After aeration, the seeds were washed in distilled water and the oat repression test was carried out in the usual manner. At the end of 96 hours the mean angle of seedlings treated in this way was 75°, which is not appreciably different from that of untreated controls. Seedlings that remained in 2,3,6-trichlorobenzoic acid, on the other hand, showed a mean angle of 43° at the end of 96 hours.

Additional information regarding the behavior of oat shoots treated with 2,3,6-trichlorobenzoic acid is obtained if the oat repression test is terminated at 56, 72, 84 or 96 hours, and a comparison of the mean angles obtained at these times is made. Such experiments were carried out in 1×10^{-5} M 2,3,6-trichlorobenzoic acid. At 56 hours no noticeable change was found in the included angle. However, the two experiments terminated at 72 hours showed considerable repression of the geotropic response. Moreover, additional repression occurred between 84 and 96 hours. The extent of repression, during the first 96 hours at least, increases with length of time the seedlings are exposed to 2,3,6-trichlorobenzoic acid (fig 7).

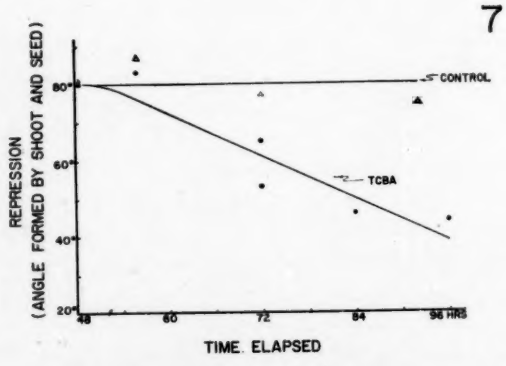


FIG. 7. Repression of the geotropic response of Avena shoots at various times during the oat repression test carried out in 1×10^{-5} M 2,3,6-trichlorobenzoic acid.

Both 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid affected the normal geotropic response in barley, oats and other seedlings. This property was not observed in seedlings treated with 2,4,5-trichlorobenzoic and 3,4-dichlorobenzoic acids. The evidence suggests that chlorination of the benzene ring at the 2 and 6 positions confers the ability to repress the geotropic response. A number of benzene and benzoic acid derivatives, variously substituted in the 2 and 6 positions, were tested to determine if substituents other than chlorine in these positions cause repression of the geotropic response. The compounds tested were: 2,6-diethylaniline, 2,4,6-trihydroxybenzoic, 2,4,6-trimethylbenzoic, 2,4,6-trinitrobenzoic, tetrachlorophthalic, 2-nitro-6-chlorobenzoic, 5,6-dimethoxy-2-formylbenzoic, 2,4,6-trimethyl-3-bromobenzoic and pentachlorobenzoic acids, and ethyl hydrogen tetrachlorophthalate. No repression of the geotropic response was found in the shoots of oat seedlings treated with any of these compounds.

Failure of barley shoots to assume the normal upright position when treated with 2,3,6-trichlorobenzoic acid, is not a response restricted to the dark. Similar disturbance in shoot growth was exhibited in the light. Therefore, a number of other species including cucumber, oats, flax, corn, rice and timothy was tested to determine the effect of treatment with 2,3,6-trichlorobenzoic acid on the phototropic response of shoots. The seedlings were grown, prior to their exposure to unilateral light, in levels which elicited slight to pronounced deviation of shoots from the normal vertical growth (5×10^{-6} M to 1×10^{-4} M). Only shoots of corn and flax responded positively to unilateral light after treatment with 2,3,6-trichlorobenzoic acid, and then only at levels of 1×10^{-5} M and lower. However, the response was not complete in these two species. Considerable irregularity was found in some shoots after 24 hours in unilateral light even though the seed was believed to be uniform.

Thus, seedlings treated with 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid fail to exhibit the normal photoropic response in addition to their previously shown abnormal geotropic response. Furthermore, the same specificity exists in these compounds in ability to impair the phototropic response as exists in ability to repress the geotropic response. This was shown when benzene and benzoic acid derivatives variously substituted in the 2 and 6 positions (listed above) were again tested to determine if substituents other than chlorine in these positions could affect the phototropic response. None of these compounds impaired the normal phototropic response of oat seedlings to unilateral light.

Summary

Shoots of oats, barley, and cucumber and various other seedlings, grown in the dark in dilute solutions of 2,3,6-trichlorobenzoic acid or 2,6-dichlorobenzoic acid, failed to assume the normal vertical position. This was presumed to be due to repression of the geotropic response. Other plant species responded similarly to these two acids.

A method was developed whereby a quantitative relationship was established between concentration of 2,3,6-trichlorobenzoic acid or 2,6-dichlorobenzoic acid and extent of repression of the geotropic response of shoots of oats. The former acid was shown to be much more active in this test. Of the two widely studied growth regulators, 2,4-dichlorophenoxyacetic acid and 2,3,5-triiodobenzoic acid, only the latter was found to be capable of interference with the geotropic response.

The phototropic response of seedlings of a number of species was also similarly impaired when treated with 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid.

A structural requirement, which appears to be associated with positions 2 and 6 of the benzene ring, for activity in the repression of the geotropic and phototropic responses was demonstrated; among the variously substituted benzoic acid derivatives tested, only 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid were active.

The author wishes to thank Dr. A. G. Norman for his advice and encouragement throughout these studies.

Literature Cited

1. Bentley, J. A. Growth regulation effect of certain organic compounds. Nature 165: 449. 1950.
2. Jones, R. L., Metcalfe, T. P. and Sexton, W. A. Relationship between the constitution and the effect of chemical compounds on plant growth IV. Jour. Sci. Food Agr. 5: 32–38. 1954.
3. Koepfli, J. B., Thimann, K. V. and Went, F. W. Phytohormones: structure and physiological activity I. Jour. Biol. Chem. 122: 763–780. 1938.
4. Leopold, A. C. Auxins and Plant Growth. Univ. of Calif. Press, Berkeley 1955.
5. Muir, R. M., Hansch, C. H. and Gallup, A. H. Growth regulation by organic compounds. Plant Physiol. 24: 359–366. 1949.
6. Thimann, K. V. The role of *ortho*-substitution in the synthetic auxins. Plant Physiol. 27: 392–404. 1952.
7. Veldstra, H. Researches on plant growth substances IV. Enzymologia 11: 97–137. 1944.
8. Veldstra, H. Researches on plant growth regulators XXI. Rec. trav. chim. 71: 15–32. 1952.
9. Wardlaw, C. W. Action of triiodobenzoic and trichlorobenzoic acids in morphogenesis. New Phytologist 52: 210–217. 1953.
10. Zimmerman, P. W. and Hitchcock, A. E. Growth-regulating effects of chlorosubstituted derivatives of benzoic acid. Contrib. Boyce Thompson Inst. 16: 209–213. 1951.

EFFECTS OF GROWTH-REGULATING SUBSTANCES ON THE CARBON DIOXIDE EVOLUTION AND POST-HARVEST RIPENING OF TOMATOES[1,2]

RICHARD T. HARTMAN

Department of Biological Sciences, University of Pittsburgh, Pittsburgh 13, Pennsylvania

In recent years considerable attention has been given to the effects of both natural and synthetic growth-regulating substances on the physiology of plants or detached plant parts. Several investigators have reported success in increasing the rate of maturation or ripening of a relatively large number of different fruits. However, up to the present time, no reports have appeared which claim satisfactory results in accelerating the post-harvest ripening of the fruit of the tomato (*Lycopersicon esculentum* Mill.) with the use of growth regulators. It was the purpose of the present investigation to study the effect of several growth-regulating substances on the ripening rate, internal constituents, and carbon dioxide production of tomatoes harvested in the mature-green stage of development.

Mitchell and Marth (28) demonstrated that the ripening time of detached fruits of bananas, apples, and pears was reduced considerably as a result of treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) but that the ripening process in tomatoes was not accelerated. Sprays of alpha-naphthaleneacetic acid applied to prevent preharvest drop were reported by Gerhardt and Allmendinger (15) to bring about an increased rate of maturation in Delicious apples and Bartlett pears. It was further demonstrated that the soluble-pectin content and the respiratory rate were much greater in sprayed than in control fruits. Batjer and Moon (2) also demonstrated a stimulation of maturation of four apple varieties with naphthaleneacetic acid. Treatment of harvested pears with 2,4-D when applied before onset of the climacteric, resulted in increased rates of ripening, respiration, and pro-

[1] Received revised manuscript August 4, 1958.

[2] This material was originally presented as a doctoral dissertation at the University of Pittsburgh, under the direction of Dr. Erston V. Miller.

duction of ethylene as shown by Hansen (17). Increased rates of respiration for peaches following treatment with 2,4-D were reported by Southwick (36), and by Smock and Gross (35) for apples treated with naphthaleneacetic acid. A delay of four to six weeks in the maturation of Montmorency cherries following monthly or semi-monthly applications of 2,4-D was reported by Zielinski and Garren (43).

Changes in constituents of tomato fruits as they ripen have been studied by many investigators. Literature relating to pectic changes and the enzymatic processes involved has been summarized by Kertesz (22). Pentzer and Heinze (31) have reviewed the general subject of post-harvest physiology of fruits, including the effects from the application of growth regulating substances.

Respiration of the tomato fruit has naturally received considerable attention. Gustafson (16) showed that there is an increase in carbon dioxide production accompanying the initial stages of coloring and that this is followed by a decrease as coloring continues. Singh and Mather (33) suggested that senescence is initiated by an accumulation of carbon dioxide in the fruit tissues which is followed by a reduced resistance of the superficial tissues to gases. Brooks (5) showed that an increased carbon dioxide content of the internal atmosphere results when the stem scar is waxed and that the exchange of air during respiration in storage occurs almost entirely through the stem scar. This region of gaseous exchange was shown by Clendenning (8) to be located in a ring of lenticular tissue surrounding the receptacle.

Beadle (3) reported that a relationship exists between the position of a fruit on a truss and the intensity of the respiratory peak at the climacteric and that premature picking results in a reduction of the climacteric peak. Two types of respiratory patterns have been described by Walford (39). One of these, the "conventional" pattern, is characterized by a climacteric rise in carbon dioxide evolution during the early color changes in ripening, followed by a fall in the rate of respiration. The other pattern, termed "anomalous," lacks the climacteric rise and shows instead a steady rate of respiration during the ripening period. The work of Clendenning (6, 7) demonstrated that the respiratory pattern could be changed by waxing the stem scar and that this change was reversible within limits. Fruits on the plant were shown to exhibit the same respiratory pattern as those in storage and also to have the area of gaseous exchange localized at the stem end. More recently (41, 42) respiration patterns and ethylene production have been studied in tomatoes in relation to ripening time.

Methods and Materials

Ripening Rate: Tomato fruits used throughout this work came from two main sources over a period of four years. During the winter and spring months, southern- and western-grown fruits, harvested and shipped in the "mature-green" stage of development, were supplied by a local pre-packaging firm. Throughout the summer months, fruits in the same stage of ripeness were obtained from a local grower. Locally grown tomatoes were of the Rutgers variety.

Fruits showing any indication of ripening were discarded at the beginning of the experiment and the remaining green fruits were divided into lots carefully matched as to size and, as far as possible, degree of maturity. The number of lots used varied with the experiment and depended upon the number of compounds and different concentrations of these compounds used in any one experiment. In the various experiments the size of the lots ranged from 20 to 60 fruits.

Eleven different compounds were tested in preliminary experiments at concentrations of 50, 100, 500 and 1,000 ppm to determine their effect on the ripening rate of green tomato fruits. These were 2,4-dichlorophenoxyacetic acid (2,4-D); 2,4,6-trichlorophenoxyacetic acid (2,4,6-T); triethanolamine salt of 2,4,6-trichlorophenoxyacetic acid; *para*-chlorophenoxyacetic acid; *ortho*-chlorophenoxyacetic acid; alpha (*ortho*-chlorophenoxy) propionic acid; alpha (*para*-tertiary-butylphenoxy) propionic acid; alpha naphthaleneacetic acid; 2-naphthoxyacetic acid; 3-indolepropionic acid; and ammonium thiocyanate. In addition, 2,4,6-T in concentration of 100, 500 and 1,000 ppm was used in combination with 1,000 ppm ammonium thiocyanate.

Aqueous solutions of these growth-regulating substances, with the exception of ammonium thiocyanate and the triethanolamine salt of 2,4,6-T, were prepared using Carbowax 4,000 as a carrier (27). Two % Carbowax was normally sufficient with concentration of 500 ppm but, with concentrations of 1,000 ppm, 3 % Carbowax was required to prevent precipitation of the growth regulator. Ammonium thiocyanate and the triethanolamine salt of 2,4,6-T were applied in water solution without Carbowax.

In preliminary experiments, solutions of growth-regulators were sprayed on the fruits with an aerosol sprayer. These fruits were spread out on tables to dry and allowed to ripen under room conditions. In later experiments, tomatoes were treated by immersing them in the growth-regulator solutions for a period of three minutes; they were then permitted to drain and dry before being placed under ripening conditions or in respiration chambers.

Lots were examined at 24-hour intervals and notes made as to general appearance, condition, and stage of ripeness. The fruits were considered "ripe" when they had attained a uniformly red color. Decay was not a major problem and seldom occurred until after the fruit had ripened. In the preliminary study, only 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) and ammonium thiocyanate appeared to be effective in stimulating ripening of green tomatoes. All subsequent tests were confined to these compounds.

Using these two compounds, 7 different experiments were carried out on tomatoes obtained from the following areas; Experiment I, Pittsburgh, Pennsylvania; Experiment II, Tracy, California; Experi-

ment III, Bradenton, Florida; Experiment IV, Ruskin, Florida; Experiment V, Brownsville, Texas; Experiment VI, Pittsburgh, Pennsylvania; and Experiment VII, Pittsburgh, Pennsylvania.

The fruits of Experiments I to IV were permitted to ripen under conditions of room temperature without any attempt to control humidity. In Experiments V to VI temperature was maintained at 20° C (68° F) and relative humidity at 90 % by keeping the fruits in an Aminco Climitizer during the ripening period. Fruits of Experiment VII were ripened in a constant temperature cabinet at 20° C (68° F) but without humidity control.

ANALYSIS OF CONSTITUENTS: Two different sampling methods were used in analyzing the ripened fruits from the various experiments. In Experiments II, III, and IV (table I) one analysis was made on each lot. The green control lot was analyzed upon receipt of the fruit. For the other lots, analyses were carried out after most of the fruits had ripened. Over-ripe and unripe fruits were discarded during preparation of the sample. In Experiments V and VI (table II), two separate analyses were carried out on each lot of ripened fruit. The 1st of these was made when about one half of the fruits from each lot had ripened. The remaining fruits were permitted to continue ripening and the 2nd analysis carried out when all fruits were ripe. It was believed that this method of sampling permitted a comparison of fruits of similar physiological age in each lot. In Experiment V the fruits treated with 2 % Carbowax ripened so slowly that only one analysis was possible. This was made on the entire lot at about the same time that the 2nd analysis was being made on other lots.

Ascorbic acid content of the fruits was determined by the 2,6-dichlorophenolindophenol visual titration method as recommended by the Association of Vitamin Chemists (1). A composite sample was prepared by removing thin segments from opposite sides of each fruit of the lot.

For analysis of pH, dry weight, and total soluble solids, larger segments were removed from the same fruits and were ground in a Waring blendor. For dry weight determinations the tissue was dried in a drying oven at 65° C under a vacuum of 25 to 28 inches of mercury. Total soluble solids were determined with a Brix hydrometer on the clear juice obtained after filtering the ground tissue through several layers of cheesecloth.

MEASUREMENT OF RESPIRATION RATE: Studies of the effect of 2,4,6-T on the respiration rate of ripening tomato fruits were carried out in four experiments conducted during two successive summers. In each of these experiments daily records were kept on 12 individual fruits, 6 untreated and 6 treated. The treatment consisted of dipping the tomatoes in an aqueous solution containing 500 ppm of 2,4,6-T in 2 % Carbowax. Locally-grown fruits were used in all respiration studies. Since fermentative metabolism is negligible in the ripening fruits, and the tissue pH sufficiently low to insure against changes in carbon dioxide retention, determination of rates of carbon dioxide evolution were considered satisfactory as a measure of respiratory activity for the ripening fruits.

The carbon dioxide evolved from these fruits was determined by a method described by Denny (11). This is a simplified procedure (Denny's Method II) in which the fruits are placed in individual jars over 50 ml of 2 *N* NaOH. In this method, oxygen consumed in the process of respiration is replaced by diffusion from the outside air through a T-tube extending through the lid of the respiration jar. Twelve fruits, 6 treated with 500 ppm 2,4,6-T and 6 untreated, were used at one time, together with two jars without fruits. The latter served as controls on the CO_2 absorbed from the atmosphere during the respiration period. The 14 jars were put on a rack and the entire apparatus placed in a constant temperature cabinet at 20° C.

The amount of CO_2 absorbed by the NaOH in each jar was determined daily by titration against 0.5 *N* HCl, for the preceding 24-hour period. The stage of ripeness of the fruits was recorded daily and CO_2 determinations were continued to include the 2nd 24-hour period following the development of a full ripe color. After the CO_2 determination on the 2nd day, the ripe fruits were transferred from the respiration jars to waxed paper containers and frozen at −20° C for subsequent determination of pectin-methylesterase activity.

MEASUREMENT OF PECTIN-METHYLESTERASE ACTIVITY: Fruits from two of the respiration experiments were tested for activity of the pectin-methylesterase enzyme to determine whether activity of the enzyme was affected by treatment with 2,4,6-T. The method of enzyme preparation and measurement was essentially that described and employed by Hills and Mottern (20).

The substrate, consisting of 0.5 % citrus pectin (Pectin, N.F. sold by California Fruit Growers Exchange), 0.05 M sodium chloride, and 0.002 M sodium oxalate was pipetted into a beaker in a 25° C water bath over a magnetic stirrer. Normally 100 ml of the substrate was sufficient but for fruits with very active pectin-methylesterase 200 ml was used. The pH of the substrate was adjusted to approximately 7.3 with 0.02 *N* NaOH and the solution stirred constantly. One milliliter of crude enzyme extract was added and the time noted when the needle of the meter passed pH 7.0. For a 15-minute period the reading was maintained at 7.0 ± 0.1 by dropwise addition of 0.02 *N* NaOH. Readings of the burette were recorded at intervals of one minute and the results expressed in milliequivalents of sodium hydroxide used during the 15-minute reaction period.

RESULTS

Of the 11 compounds tested only two, 2,4,6-T and ammonium thiocyanate, proved to be effective in shortening the ripening time of green tomato fruits. One compound, alpha-naphthaleneacetic acid, seemed

to extend the ripening period when applied at concentrations of 100, 500 or 1,000 ppm. No additional studies were made with this compound.

With either 2,4,6-T or ammonium thiocyanate, concentrations of 500 or 1,000 ppm gave maximum effects in accelerating the rate of ripening. In all experiments 2,4,6-T at a concentration of 500 ppm was superior to 1,000 ppm of ammonium thiocyanate. Combinations of these two compounds were no more effective in accelerating ripening than were the same concentrations used separately. The results presented in figures 1 and 2 are typical of the results obtained in 6 ripening experiments. In the various experiments the two substances were generally effective in reducing the ripening time of mature-green tomatoes by one to four days.

Results indicated that the compounds showed little or no effect as accelerators of ripening on fruits which colored soon after treatment, but were effective when ripening did not occur for at least 6 days following application of the accelerators. In one experiment an increase in ripening time did not appear until 13 days after treatment with 2,4,6-T or 14 days with ammonium thiocyanate. After this period, ripening was increased from one to four days over the control with the former compound and from one to two days with the latter. It thus appears that a relationship exists between the stage of maturity of a fruit at the time of treatment and the response to these accelerating compounds.

The effect of Carbowax 4000 may be seen in the results shown in figure 1. It will be noted here that the ripening of fruits treated with 2% Carbowax only was delayed during the early part of the experimental period but that, in spite of this, toward the end of the ripening period this lot was at essentially the same stage of ripeness as the untreated control. Similar results were found in other experiments.

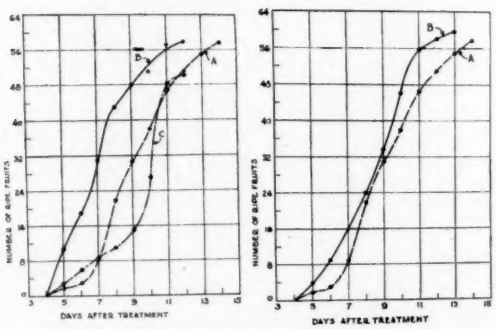


FIG. 1 (*left*). Ripening rates of mature-green tomato fruits. A. Untreated controls. B. Treated with 500 ppm 2,4,6-T in 2% Carbowax. C. Treated with 2% Carbowax.

FIG. 2 (*right*). Ripening rates of mature-green tomato fruits. A. Untreated controls. B. Treated with 1000 ppm ammonium thiocyanate.

In several experiments, despite careful selection at the beginning, fruits were found in control lots which failed to ripen within the time limit of the experiment. These had the characteristics of immature tomatoes as described by Lutz (25). Immature tomatoes also occurred in the treated lots but these fruits always ripened within the available time, indicating a stimulating effect by the growth-regulating substances.

An experiment was designed to determine whether there was a greater response to 2,4,6-T by immature fruits. In this case, lots composed of tomatoes in the immature-green stage of development were used along with mature-green fruits. Immature fruits were characterized by a whitish-green color, an uneven surface, and a greater firmness than the mature-green tomatoes. The results indicated that the ripening rate of immature fruits was accelerated about two and one half days by 2,4,6-T, but apparently not to a greater extent than in the mature-green fruits.

There was little difference in appearance between fruits ripened after application of the accelerators and those ripened without treatment, except for a spotty appearance on the surface of some of the early-ripening fruits in lots treated with 2,4,6-T in Carbowax. Spotted areas were yellow to green in appearance and were confined to the surface layer. Tissue immediately beneath these spots was a normal red color. Hansen (17) has described a similar mottled appearance in pears treated with concentrations of 100 ppm and higher of 2,4-D.

Examination of the mottled tomatoes showed the spots to be associated with heavy deposits of Carbowax on the surface. Apparently the retardation of coloring in these areas was caused by the presence of rather high concentration of 2,4,6-T held in the heavy deposits of Carbowax since fruits treated with Carbowax alone showed even coloring of the fruit surface The mottled effect, however, was not seen on treated fruits which ripened slowly but appeared almost entirely in those fruits which ripened within 7 to 10 days after treatment.

Results of analyses of the fruits from the various experiments for pH, soluble solids, and ascorbic acid indicated that in general, the composition of fruits ripened after treatment with 2,4,6-T or ammonium thiocyanate was not different from that of fruits ripened without the chemical treatment. Values from several of the experiments are presented in tables I and II.

The lack of effect upon the constituents of tomatoes as a result of treatment with 500 ppm 2,4,6-T was further demonstrated with the pectin-methylesterase system. Individual fruits, both treated and untreated, showed wide variations in enzyme activities when expressed as milliequivalents of bonds hydrolyzed. Values ranged within one study from 0.079 to 0.567 and from 0.346 to 0.518 in another. For 16 treated and 16 untreated fruits the average values from the three studies were 0.390 and 0.398 milliequivalents respectively.

TABLE I

EFFECT OF GROWTH-SUBSTANCES ON THE CONSTITUENTS OF TOMATO FRUITS *

METHOD OF TREATMENT	pH		DEGREES BRIX		ASCORBIC ACID (MG/100 G)	
	EXPT III	EXPT IV	EXPT III	EXPT IV	EXPT III	EXPT IV
Green controls	4.15	4.38	4.55	5.00	9.5	10.2
Untreated ripe controls	4.25	4.35	4.75	4.92	10.7	10.6
2 % Carbowax 4000	4.20	4.35	4.63	5.16	10.2	12.0
500 ppm 2,4,6-T	4.15	4.35	4.63	4.95	10.5	11.1
1000 ppm NH_4CNS	4.22	4.35	4.47	4.92	9.7	12.0
1000 ppm NH_4CNS + 100 ppm 2,4,6-T	4.26	4.40	4.45	4.66	10.4	12.4
1000 ppm NH_4CNS + 500 ppm 2,4,6-T	4.25	4.35	4.50	4.75	11.4	13.6
1000 ppm NH_4CNS + 1000 ppm 2,4,6-T	4.24	4.38	4.65	4.86	11.1	14.5

* Analysis of entire lot.

Plotting the daily carbon dioxide evolution from each fruit of the four experiments permitted an examination of the individual respiration patterns. The majority of the fruits followed the "conventional" respiration pattern as defined by Walford (39). This pattern was found in all fruits of one experiment, in all but one fruit of two experiments, and in 9 fruits of the 4th experiment. The remaining fruits (see fruit number 3, figure 3 as an example) failed to show a climacteric rise during the color changes of ripening. These appeared to follow the type of respiration drift designated by Walford as "anomalous."

Walford has indicated that the "conventional" fruit is characteristic of the late spring and summer seasons while the "anomalous" type occurs in the autumn, winter, and early spring. He points out, however, that either type may actually be present in all seasons. Clendenning (6) states that both types have been found at all seasons of the year as well as

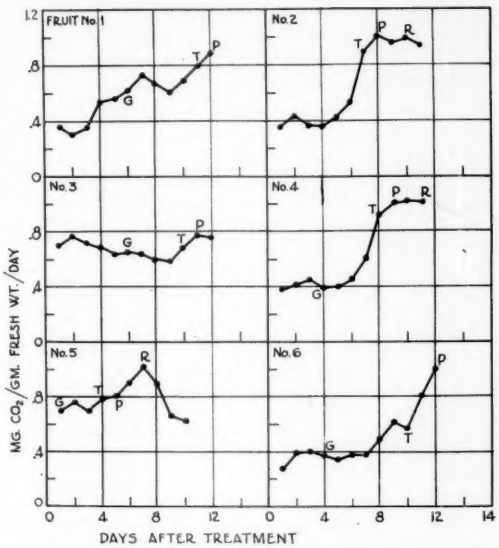


FIG. 3. Respiration Experiment II. Rate of carbon dioxide evolution during ripening of untreated tomato fruits. (G, green; T, turning; P, pink; R, red.)

under different light intensities in the same season. Intermediate types which appear sporadically with no apparent association with seasons also have been reported.

Results from one of the four experiments (Experiment II) are shown in figures 3 and 4. The irregularity of some of the curves in the early part of the experiments is in agreement with the findings of Walford who speaks of this as an "initial effect" and believes it to be related to changes in starch content of fruits isolated from the plant at a stage when starch was abundant.

It should be noted that, with fruits showing the "conventional" respiration drift, the climacteric rise appeared earlier in treated than in untreated fruits, coincident with the shortening of the ripening period.

TABLE II

EFFECT OF GROWTH-SUBSTANCES ON THE CONSTITUENTS OF TOMATO FRUITS *

METHOD OF TREATMENT	pH		DEGREES BRIX		ASCORBIC ACID (MG/100 G)		PERCENT MOISTURE	
	1ST ANALYSIS	2ND ANALYSIS	1ST ANALYSIS	2ND ANALYSIS	1ST ANALYSIS	2ND ANALYSIS	1ST ANALYSIS	2ND ANALYSIS
Experiment VI								
Untreated, green	4.72	...	5.57	...	16.8	...	92.7	...
Untreated, ripe	4.35	4.30	5.75	5.51	17.2	13.5	93.2	...
2 % Carbowax	4.25	4.26	5.80	5.60	16.4	13.1	93.8	...
500 ppm 2,4,6-T	4.30	4.30	5.86	5.47	18.5	14.9	94.0	...
1000 ppm NH_4CNS	4.35	4.40	5.80	5.61	17.6	13.7	93.4	...
500 ppm 2,4,6-T + 1000 ppm NH_4CNS	4.30	4.30	5.71	5.62	18.5	14.8	93.6	...

* Lots analyzed in two samples.

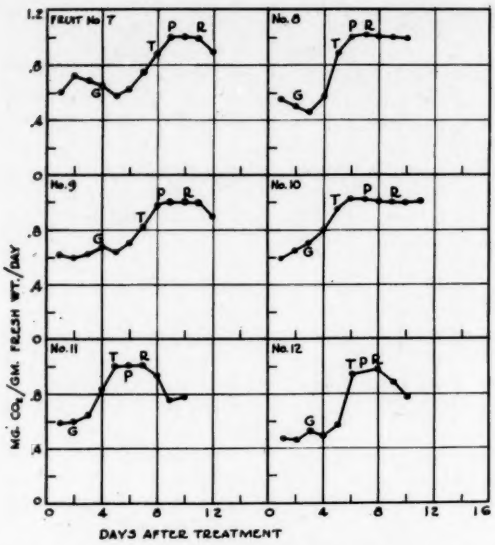


FIG. 4. Respiration Experiment II. Rate of carbon dioxide evolution during ripening of tomato fruits treated with 500 ppm 2,4,6-T in 2% Carbowax. (G, green; T, turning; P, pink; R, red.)

The peak of the climacteric for the treated fruits was, however, not higher than that of the untreated fruits. In fact, when the values for the climacteric peaks for the 6 treated fruits of each of the four experiments are averaged they do not differ from the average of the climacteric peak values for the untreated fruits.

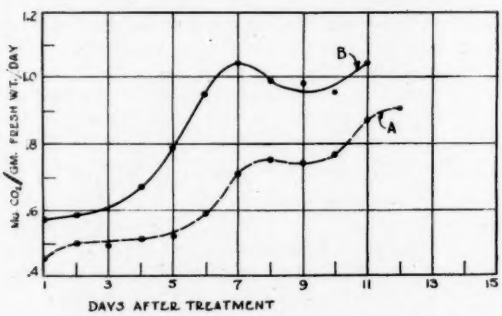


FIG. 5. Respiration Experiment II. Rate of carbon dioxide evolution expressed as mean values of 6 fruits. A. Untreated controls. B. Treated with 500 ppm 2,4,6-T in 2% Carbowax.

Figure 5 presents the results of the respiration Experiment II in terms of the mean daily carbon dioxide output for the six fruits of each experimental lot. A definite rise in carbon dioxide evolution from the entire lot, as a result of treatment with 500 ppm 2,4,6-T, is indicated by these results. It must be recognized this increase in carbon dioxide output occurs in spite of the fact that the height of the individual climacteric peaks was not increased. This greater evolution of carbon dioxide apparently is the result of the earlier appearance of the climacteric rise in the fruit whose ripening was accelerated by the 2,4,6-T.

DISCUSSION

The two compounds which were shown to accelerate ripening of green tomatoes do not possess the properties customarily associated with active growth-regulating compounds. The relative ineffectiveness of 2,4,6-T in inducing cell elongation and curvature, stimulating root formation, or bringing about toxic responses has been pointed out by several investigators (19, 23, 24, 30, 38, 40). More recently, however, Thimann (37) has shown that this compound shows auxin activity in the pea test. Ammonium thiocyanate, although not considered as an auxin, has been shown to be effective in forcing early sprouting of potatoes (9, 10), in enhancing red color formation in apples (12, 13), in activating 2,4-D (21), and as a toxic agent useful in herbicidal treatments (32). It is apparent, however, that in growth-modifying ability, both of these compounds differ greatly from 2,4-D.

Mitchell and Marth (28) suggested that the accelerating action of 2,4-D on the ripening time of bananas, apples, and pears may be related to an increased diastatic activity and pointed out that tomatoes lack an appreciable starchy reserve. Emmert and Southwick (14), in studies of the effects of 2,4-D and the methyl ester of naphthaleneacetic acid on tomatoes, concluded that neither substance consistently affected respiration of mature green fruits. In consideration of these points it would appear that the mechanisms responsible for accelerating ripening in tomato fruits by 2,4,6-T and ammonium thiocyanate are distinct from the mechanisms concerned with typical growth-regulator responses.

In the work reported here, Carbowax 4000 was observed to retard ripening of tomatoes during the early stages of the experimental period although lots treated with this material eventually contained as many ripe fruits as control lots. Since 2,4,6-T was applied in Carbowax solutions it may be assumed that the delaying effect of the carrier was encountered here also. Apparently the influence of 2,4,6-T was great enough to overcome the temporary delaying effects of Carbowax and, in addition, to show a further acceleration. If a suitable carrier could be found which caused no retarding effects, or if 2,4,6-T could be applied alone, a greater acceleration might result than that demonstrated in this investigation. The retarding effects of alpha-naphthaleneacetic acid, although observed only in preliminary experiments, merit further study. It may well be that further studies will reveal that by application of proper accelerating or retarding compounds, mature-green tomatoes may be ripened almost at will.

It appears from the results of this investigation that neither 2,4,6-T nor ammonium thiocyanate at concentration up to 1000 ppm cause any great changes in soluble solids, pH, ascorbic acid values, or in pectinmethylesterase activity. This is in agreement with the comments by Mitchell and Marth (29) in a review of the subject. Hansen and Hartman (18) working with ethylene, and Hansen (17) with 2,4-D have shown that for pears the greatest acceleration of ripening was obtained with fruits in which the climacteric rise in respiration had not been initiated. The present observations with tomatoes, where the greatest effects on the ripening rate of tomatoes occurred with fruits treated at least 6 days before ripening, appear to be in line with these results.

The results obtained from the four respiration experiments reported here are of interest since it is generally conceded that respiratory activity is perhaps the best single index of the metabolic state of a plant organ. It was demonstrated that while a greater amount of carbon dioxide was evolved during the ripening period by fruits treated with 2,4,6-T, the climacteric peaks of individual fruits were not increased above those of untreated fruits. The difference in total carbon dioxide production seemed to result mainly from the earlier appearance of the climacteric rise in the individual, treated fruits. These results are not in agreement with the statement made by Biale (4) in reviewing the effects of growth-regulating substances on the respiration of ripening fruits. Biale concluded, "whenever growth regulating substances affect ripening they give a higher peak value for carbon dioxide production than the control fruit. Ethylene treatment, on the other hand, results in most cases in a shift of the time axis but not in any increase of the climacteric peak value." From the results of the present investigation it would appear that the influence of 2,4,6-T on respiration was more characteristic of ethylene than of the typical growth-regulator. The various investigations on which Biale's comments were based were not carried out with individual fruits but, on lots consisting of a number of fruits. It is possible that the resultant respiration pattern of these lots obscured the patterns of the individual fruits.

It must be recognized that an increase in carbon dioxide production is an indication of increased metabolic activity within the fruit. This in turn implies a more rapid utilization of storage constituents and therefore perhaps a shortened storage life for the fruit. More extensive studies are necessary to determine what effect these ripening accelerators have on the keeping quality of tomato fruits.

The mechanisms whereby ripening processes are affected by these compounds is not known. It has been shown (17) that treatment of pears with 2,4-D resulted in increased ethylene production by the fruits. However, Mitchell and Marth concluded (26) that 2,4-D was antagonistic to the ripening effects of ethylene on bananas. Maleic hydrazide apparently counteracts some of the stimulating effects of growth substances on apples when applied simultaneously (34). Ethylene is known to accelerate the ripening of many fruits including tomatoes and is, in fact, a normal metabolite of many ripening fruits. Whether or not 2,4,6-T and ammonium thiocyanate stimulate the production of ethylene in tomatoes must be determined by further study. A study of this type would be of importance since it may lead to a better understanding of the mechanisms of action of both growth-regulating substances and ethylene on ripening processes and to a knowledge of possible relationships between their actions.

It should be noted that under present Food and Drug Administration regulations, these chemicals may not be used in the commercial ripening of tomatoes or other fruits.

Summary

Studies were made of the effects of 11 synthetic growth substances on the post-harvest ripening of mature-green tomatoes. Two compounds, 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) and ammonium thiocyanate, were shown to be effective in shortening the ripening time of green tomatoes by a period of one to four days.

The accelerating substances were most effective when applied at least 6 days before the "red" stage of ripeness. This appears to correspond to the period before onset of the climacteric rise in respiration.

Application of the accelerating substances did not affect constituents of ripened fruits as shown by analyses of pH, soluble solids, ascorbic acid, and pectinmethylesterase activity.

A study of the respiratory patterns of individual fruits treated with 500 ppm 2,4,6-T and of untreated fruits revealed that, while the climacteric rise in carbon dioxide evolution appeared earlier in the treated fruits, their climacteric peaks were not higher than those of untreated fruits. As a group, in each of four experiments, the treated fruits evolved more carbon dioxide than did controls during the ripening period. This was apparently a result of the earlier appearance of the climacteric rise in fruits accelerated by the 2,4,6-T.

The author wishes to express his appreciation to The Dow Chemical Company for supplying some of the growth regulating substances used in this study and to Koppers Company, Inc. for ammonium thiocyanate and for making available funds used for the purchase of several lots of tomatoes.

Literature Cited

1. Association of Vitamin Chemists, Inc. Methods of Vitamin Assay. Interscience Publishers, New York 1947.
2. Batjer, L. P. and Moon, H. H. Effects of naphthaleneacetic acid spray on maturity of apples. Proc. Amer. Soc. Hort. Sci. 46: 113–117. 1945.
3. Beadle, N. C. W. Studies in the growth and respiration of tomato fruits and their relationship to carbohydrate content. Australian Jour. Expt. Biol. and Med. Sci. 15: 173–189. 1937.
4. Biale, J. B. Postharvest physiology and biochemistry of fruits. Ann. Rev. Plant Physiol. 1: 183–206. 1950.

5. Brooks, C. Some effects of waxing tomatoes. Proc. Amer. Soc. Hort. Sci. 35: 720. 1938.
6. Clendenning, K. A. Studies of the tomato in relation to its storage. II. The effect of altered internal atmosphere upon the respiratory and ripening behavior of tomato fruits stored at 12.5° C. Can. Jour. Research, C. 19: 500–518. 1941.
7. Clendenning, K. A. The respiratory and ripening behavior of the tomato fruit on the plant. Can. Jour. Research, C. 20: 197–203. 1942.
8. Clendenning, K. A. Growth studies of normal and parthenocarpic tomato fruits. Can. Jour. Research, C. 26: 507–513. 1948.
9. Denny, F. E. Hastening the sprouting of dormant potato tubers. Amer. Jour. Bot. 13: 118–125. 1926.
10. Denny, F. E. Second report on the use of chemicals for hastening the sprouting o fdormant potato tubers. Amer. Jour. Bot. 13: 386–396. 1926
11. Denny, F. E. Suggested methods for measuring the carbon dioxide production of a large number of simultaneous samples. Contrib. Boyce Thompson Inst. 15: 141–151. 1948.
12. Dustman, R. B. and Duncan, I. J. Red color in apples. Science 90: 233. 1939.
13. Dustman, R. B. and Duncan, I. J. Effect of certain thiocyanate sprays on foliage and fruit in apples. Plant Physiol. 15: 343–348. 1940.
14. Emmert, F. H. and Southwick, F. W. The effect of maturity, apple emanations, waxing, and growth regulators on the respiration and red color development of tomato fruits. Proc. Amer. Soc. Hort. Sci. 63: 393–401. 1954.
15. Gerhardt, F. and Allmendinger, D. F. The influence of alpha-naphthaleneacetic acid spray on the maturity and storage physiology of apples, pears, and sweet cherries. Jour. Agric. Research 73: 189–206. 1946.
16. Gustafson, F. G. Growth studies on fruits. Respiration of tomato fruits. Plant Physiol. 4: 349–356. 1929.
17. Hansen, E. Effect of 2,4-dichlorophenoxyacetic acid on the ripening of Bartlett pears. Plant Physiol. 21: 588–592. 1946.
18. Hansen, E. and Hartman, H. Effect of ethylene and certain metabolic gases upon respiration and ripening of pears before and after cold storage. Plant Physiol. 12: 441–454. 1937.
19. Hansch, C. and Muir, R. M. The ortho effect in plant growth-regulators. Plant Physiol. 25: 389–393. 1950.
20. Hills, C. H. and Mottern, H. H. Properties of tomato pectase. Jour. Biol. Chem. 168: 651–663. 1947.
21. Hitchcock, A. E. and Zimmerman, P. W. Activation of 2,4-D by various adjuvants. Contrib. Boyce Thompson Inst. 15: 173–193. 1948.
22. Kertesz, Z. I. The Pectic Substances. Interscience Publishers, New York 1951.
23. Kraus, E. J. and Mitchell, J. W. Growth-regulating substances as herbicides. Bot. Gaz. 108: 301–350. 1947.
24. Leaper, J. M. F. and Bishop, J. R. Relation of halogen position to physiological properties in the mono-, di-, and trichlorophenoxyacetic acids. Bot. Gaz. 112: 250–258. 1951.
25. Lutz, J. M. Maturity and handling of green-wrap tomatoes in Mississippi. U. S. Dept. Agric. Circ. 695. 1944.
26. Marth, P. C. and Mitchell, J. W. Apparent antagonistic effects of growth-regulators. Bot. Gaz. 110: 514–518. 1949.
27. Mitchell, J. W. and Hamner, C. L. Polyethylene glycols as carriers for growth-regulating substances. Bot. Gaz. 105: 474–483. 1944.
28. Mitchell, J. W. and Marth, P. C. Effects of 2,4-dichlorophenoxyacetic acid on the ripening of detached fruit. Bot. Gaz. 106: 199–207. 1944.
29. Mitchell, J. W. and Marth, P. C. Growth-regulating substances in horticulture. Ann. Rev. Plant Physiol. 1: 125–140. 1950.
30. Muir, R. M., Hansch, C. H. and Gallup, A. H. Growth regulation by organic compounds. Plant Physiol. 24: 359–366. 1949.
31. Pentzer, W. T. and Heinze, P. H. Postharvest physiology of fruits and vegetables. Ann. Rev. Plant Physiol. 5: 205–224. 1954.
32. Robbins, W. W., Crafts, A. S. and Raynor, R. N. Weed Control. Pp. 318–325. McGraw-Hill, New York 1942.
33. Singh, B. N. and Mather, P. B. Dissolved carbon dioxide and the ripening of tomatoes. Nature 137: 704–705. 1936.
34. Smock, R. M., Edgerton, L. J. and Hoffman, M. B. Inhibition of the ripening effect of certain auxins on apples with maleic hydrazide. Proc. Amer. Soc. Hort. Sci. 60: 184–192. 1952.
35. Smock, R. M. and Gross, C. R. The effect of some hormone materials on the respiration and softening rates of apples. Proc. Amer. Soc. Hort. Sci. 49: 67–77. 1947.
36. Southwick, F. W. Effect of some growth-regulating substances on the rate of softening, respiration, and soluble solids content of peaches and apples. Proc. Amer. Soc. Hort. Sci. 47: 84–90. 1946.
37. Thimann, K. V. The role of *ortho*-substitution in the synthetic auxins. Plant Physiol. 27: 392–404. 1952.
38. Thompson, H. E., Swanson, C. P. and Norman, A. G. New growth-regulating compounds. I. Summary of growth-inhibitory activities of some organic compounds as determined by three tests. Bot. Gaz. 107: 476–507. 1946.
39. Walford, E. J. M. Studies of the tomato in relation to its storage. I. A survey of the effect of maturity and season upon the respiration of greenhouse fruits at 12.5° C. Can. Jour. Research, C. 16: 65–83. 1938.
40. Wittwer, S. H. Growth substances in fruit setting. In: Plant Growth Substances. F. Skoog, ed. University of Wisconsin Press, Madison 1951.
41. Workman, M., Pratt, H. K. and Norris, L. L. Studies on the physiology of tomato fruits. I. Respiration and ripening behavior at 20° C as related to date of harvest. Proc. Amer. Soc. Hort. Sci. 69: 352–365. 1957.
42. Workman, M. and Pratt, H. K. Studies on the physiology of tomato fruits. II. Ethylene production at 20° C as related to respiration, ripening, and date of harvest. Plant Physiol. 32: 330–334. 1957.
43. Zielinski, Q. B. and Garren, R. G., Jr. Effect of 2,4-dichlorophenoxyacetic acid and naphthaleneacetic acid on increasing fruit-setting and delaying maturity of Montmorency cherries. Bot. Gaz. 113: 147–150. 1951.

STUDIES ON SULFATE UTILIZATION BY CHLORELLA PYRENOIDOSA USING SULFATE-S^{35}; THE OCCURRENCE OF S-ADENOSYL METHIONINE[1,2]

JEROME A. SCHIFF[3]

DEPARTMENT OF BOTANY, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PENNSYLVANIA

There is an abundance of nutritional evidence that higher plants and many microorganisms can use sulfate as the sole source of sulfur, but the mechanism of sulfate reduction and incorporation into organic compounds is not established. Horowitz and co-workers (9, 10, 17, 28) have investigated the intermediates of sulfate reduction in mutants of *Neurospora crassa* and have proposed cysteic acid and cysteine sulfinic acid as intermediates between sulfate and cysteine. On the basis of newer evidence which indicates that cysteine sulfinic acid is equivalent to cysteic acid and sulfite in Neurospora auxotrophs, Horowitz (11) has revised these earlier proposals. With the finding that all Neurospora mutants which are blocked between thiosulfate and cysteine can utilize elemental sulfur, Horowitz now suggests that the reductive pathway is a completely inorganic one leading from sulfate through sulfite, thiosulfate and sulfide (since Neurospora spores convert elemental sulfur to sulfide) to the linkage of sulfide to a three carbon acceptor to form cysteine. Cysteine sulfinic acid is probably oxidized to cysteic acid which is then converted to sulfite for further reduction. Singer and Kearney (25) have pointed out, however, that in systems that metabolize cysteine-sulfinic acid, the oxidation of this compound to cysteic acid is a very slow reaction or is entirely absent. They speculate that the site of linkage of inorganic sulfur to a carbon chain may be through the reaction of sulfite and pyruvate to yield beta-sulfinyl pyruvate.

Cowie and his co-workers (4) have shown that cysteic acid does not compete with the incorporation of radioactive sulfate in *Escherichia coli* and, therefore, may not be an intermediate in sulfate reduction in this organism. Ragland and Liverman (18) are currently investigating the intermediary metabolism of sulfate reduction in sulfur auxotrophs of Neurospora.

[1] Received revised manuscript August 15, 1958.

[2] During part of the period in which this work was conducted, the author received support from a U. S. Public Health Service Pre-doctoral Research Fellowship. Part of the support for this research was derived from Atomic Energy Commission Grant No. AT (30-1)-1460. The work reported here was taken from a thesis submitted to the Graduate Faculty of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Present address: Dept. of Biology, Brandeis University, Waltham, Massachusetts.

The status of cystathionine as an intermediate between cysteine and methionine has also been investigated. While Horowitz et al have found this compound in certain auxotrophs of Neurospora (28), McRorie and Carlson (16) have indicated that there may be a route of conversion in Neurospora which does not involve cystathionine. Cowie has been unable to demonstrate cystathionine as an intermediate in *E. coli* by his competition techniques (4), but Lampen (12) has shown that auxotrophs of *E. coli* can utilize cystathionine as a sulfur source.

The reduction of sulfate to the level of hydrogen sulfide is highly endergonic and requires 8 electrons. By analogy to other oxidation-reduction systems one might suppose that the reduction would proceed in four steps with the transfer of two electrons per step. It is entirely possible that the inorganic species are not reduced directly, but after linkage to some carrier (such as adenylic acid) which changes the potential enough to permit reduction by compounds which ordinarily could not bring about the reaction. The free energy change in the reduction of sulfate by hydrogen may be calculated from the free energies of formation of the compounds from their elements (13):

$$S + O_2 \longrightarrow SO_4^{=} \qquad \Delta F = -176.1 \text{ kg Cal.}$$

$$S + H_2 \longrightarrow H_2S \text{ (aq.)} \qquad \Delta F = -6.52 \text{ kg Cal.}$$

Rearranging gives:

$$SO_4^{=} + H_2 \longrightarrow H_2S \text{ (aq.)} + O_2 \qquad \Delta F = +169.6 \text{ kg Cal.}$$

If one assumes that a reductant at the level of triphosphopyridine nucleotide is involved one may write (6):

$$SO_4^{=} + 4\,TPNH + 6\,H^{+} \longrightarrow H_2S + 4\,H_2O + 4\,TPN^{+} \qquad \Delta F = -208 \text{ kg Cal.}$$

If the reduction proceeded in this way, the process would be highly wasteful as far as energy is concerned. It is possible that the real reductant in the cells is closer to the potential of the sulfate-hydrogen sulfide system and that prior linkage of the inorganic species occurs to change the potentials of the sulfate —H_2S system.

Quite recently, in this connection, compounds of adenylic acid or adenosine with sulfur compounds have come into prominence. Hilz and Lipmann (7)

have described an enzyme system which brings about the activation of sulfate with ATP to yield adenosine-3′-phosphate-5′-phosphosulfate or "active sulfate" (19, 20). They have found this system in *Neurospora crassa* and in mammalian systems. Bandurski (1) has also described a similar system from yeast. The mammalian system also contains enzymes which bring about the reaction of "active sulfate" and *p*-nitrophenol to yield *p*-nitrophenol sulfate (7). Schlenk and his co-workers (23) have described a compound from yeast, thiomethyladenosine, which serves as a source of the thiomethyl group in other compounds and Schwartz and Shapiro (24) have shown that thiomethyl-adenosine serves as a source of the thiomethyl group of methionine in a mutant of *Aerobacter aerogenes*. Cantoni has demonstrated the formation of a similar compound, S-adenosyl methionine, from ATP and methionine in mammalian systems (2) which serves as a transmethylating agent, and is formed through a unique triphosphate cleavage of ATP which yields orthophosphate and pyrophosphate (3).

Nutritional studies which we have reported previously (21, 22) have shown that sulfate or cysteine will serve as the sole sulfur sources for the growth of Chlorella. Methionine apparently cannot serve as a sole sulfur source and leads to lower growth rates and chlorotic cells. We have also described experiments with methionine-S^{35} which have indicated that methionine enters the cells but that the sulfur label does not spread to compounds other than the methionine of protein to any appreciable extent. In these experiments difficulties were encountered in obtaining reproducible chromatographic patterns. The work to be described here attempts to establish conditions for radiosulfate utilization by Chlorella which are controllable and which lead to consistent chromatographic patterns.[4]

Materials and Methods

Chlorella pyrenoidosa Chick, obtained from Professor William Stepka, was grown on a modification of the medium of Hopkins (8) shown in table I. The medium was autoclaved for 40 minutes at 15 lbs/in^2. Growth took place in 2-liter flat-bottomed flasks for one to two weeks with mild aeration in a dark, temperature controlled room maintained at 26° ± 2° C; the cells were then harvested by centrifugation at 2° C, washed with cold sulfur-free medium and were resuspended in sulfur-free medium to give a final concentration of 0.1 ml of packed cells per ml. This suspension was then aerated for one half hour at room temperature before use.

One-ml aliquots were transferred to 12-ml centrifuge tubes, each equipped with a capillary aerator,

[4] At a time when the work to be described here had been completed, studies on sulfate utilization by Chlorella in the light were reported in abstract by Zweig and Hood (31). Liverman and Ragland (14) have recently reported investigations of the sulfate metabolism of the Alaska pea.

Table I

Medium for Growth of Chlorella

Substance	M/L	ml/L
KNO_3	0.01	
$MgNO_3$	0.005	
KH_2PO_4	0.004	
K_2HPO_4	0.001	
Na_2SO_4	0.005	
Ferric tartrate	1.8×10^{-5}	
Glucose	0.083	
Sodium citrate	0.0031	
Special A_5 solution *		1.0

* The constitution of Special A_5 solution is as follows: 500 ppm boron, 500 ppm manganese, 50 ppm zinc, 20 ppm copper, 50 ppm molybdenum, and 10 ppm cobalt, as the sulfur-free salts.

"Sulfur-free" medium is the above medium with Na_2SO_4, ferric tartrate, sodium citrate and glucose omitted.

for the various experiments in which radioactive sulfate was given. All experiments were carried out under ordinary laboratory lighting conditions of 25 ft-c or less.

At the end of the experimental period, the tubes were cooled in an ice bath, the cells were separated by centrifugation and were washed with 1.0 ml of cold sulfur-free medium. They were then suspended in 1.0 ml of either 1 *N* HCl or 0.1 M sodium citrate-citric acid buffer pH 5.5 and were subjected to three consecutive cycles of freezing and thawing between − 10° C and room temperature. The cell debris was centrifuged and the extract removed. The debris was then washed with another aliquot of the extracting solution and was combined with the first extract. This constitutes the "acid soluble" (AE) fraction. In one experiment the cell debris was extracted once more with boiling 80 % aqueous ethanol (v/v) followed by 30 % aqueous ethanol (v/v) and then water at room temperature. When combined these constitute the "ethanol soluble" (EE) fraction. In some experiments the cell debris containing the material insoluble in HCl was subjected to acid hydrolysis in 2.0 ml of 6 *N* HCl for four hours at 108° C. The mixture after hydrolysis constitutes the "hydrolyzable material" (HE).

Chromatography of the extracts was carried out on washed sheets (57 × 46 cm) of Whatman no. 3 filter paper. Development was by solvent descent in two different mixtures:

1st Direction: The solvent of Mason and Berg (15) was employed:

methanol (absolute)	120 ml
n-butanol	60 ml
benzene	60 ml
water (distilled)	60 ml

2nd Direction: Stepka's solvent (26) was used:

n-butanol	100 ml
water (distilled)	50 ml
acetic acid (glacial)	22 ml

Radioautography was carried out in the usual manner as described previously (21).

Paper electrophoresis employed the conditions described by Hilz and Lipmann (7). Sodium citrate-citric acid buffer pH 5.5, 0.1 M was used with a migration path of 48 cm. The separations were run either for 17 hours at 190 V at 2° C or for four hours at 300 V at 5 to 10° C.

All counting for radioactive experiments was carried out at infinite thinness with a Nuclear model 163 scaler equipped with a 1.4 mg/cm^2 mica end-window tube. Sulfate-S^{35} was obtained from Oak Ridge National Laboratory as the carrier-free isotopic compound in dilute HCl.

Column chromatography was carried out with Dowex-50 as the adsorbent as described by Tabachnick and Tarver (27). Concentrations of HCl up to 6 N were employed instead of the maximum of 3 N used by these authors and different volumes of effluent were collected in some instances.

Iodoacetamide was synthesized by a method based on the brief description given by von Braun (29).

The enzyme used in the assay of "active sulfate" was prepared by a modification of the method of de Meio et al (5). Livers from four adult rats were homogenized in a glass homogenizer in three parts of ice-cold 0.1 M phosphate buffer pH 7.0. The homogenate was then subjected to centrifugation at 100,000 × G in a Spinco preparative centrifuge for 30 minutes. The supernatant was brought to one fourth saturation with ammonium sulfate and was then centrifuged for 10 minutes at 25,000 × G. Ammonium sulfate was added to the supernatant to bring it to one half saturation. The precipitate was isolated by centrifugation and was stored at − 10° C.

For assay purposes approximately 1 g of precipitate was dissolved in 10 ml of 0.1 M phosphate buffer pH 7.0 and was dialyzed against 0.005 M phosphate buffer pH 7.0 for 24 hours, at 2° C. The contents of the dialysis sack were then used in the described experiments.

Chlorella extract for the assay of "active sulfate" was prepared as follows: a suspension of 5 g wet weight of Chlorella was suspended in sulfur-free medium as described in materials and methods. To the suspension under aeration was added enough sodium sulfate to bring the final concentration to 0.005 M. Exactly three minutes after sulfate addition enough iodoacetamide was added to bring the suspension to 10^{-2} M iodoacetamide. Aeration was continued for 20 minutes. The suspension was then chilled in an ice bath and centrifuged. After washing with sulfur-free medium, the cells were suspended in 10 ml of 0.1 M phosphate buffer, pH 7.1, containing 0.5 M cysteine/ml in order to conjugate any excess iodoacetamide, and was subjected to three consecutive cycles of freezing and thawing. This extract constitutes the "Chlorella preparation."

Experimental Results

In contrast to the slow spread of the sulfur label from methionine-S^{35} to other compounds of the acid extracts of Chlorella (21), the spread of carrier-free sulfate-S^{35} is quite rapid. One tenth of a milliliter of packed Chlorella cells can remove over 10^6 cpm of radioactive sulfate from the medium and can convert it to reduced form in less than 15 minutes. The rapidity of the sulfate incorporation process necessitated some means of control which would stop the incorporation at various stages. If sulfhydryl compounds were produced from sulfate reduction, these could react with each other oxidatively during extraction and chromatography to form all the possible disulfides and other oxidative artifacts. In order to obviate both of these experimental difficulties, iodoacetamide was used in subsequent experiments. Iodoacetamide should act in at least two ways. If any compounds of a sulfhydryl nature were formed during sulfate incorporation, iodoacetamide should react with these to produce the corresponding S-substituted acetamides which could not form oxidative artifacts of the disulfide type. Secondly, if any of the enzymes involved in sulfate reduction required free sulhydryl groups for activity, iodoacetamide might be expected to inhibit the process to some extent.

The effect of iodoacetamide at various concentrations on radiosulfate uptake into the various fractions is shown in table II. Increasing concentrations of iodoacetamide serve to increase the incorporation of the label into the acid solubles (AE) at the expense of the acid insolubles (HE). The alcohol soluble material (EE) is a small part of the total and does not change significantly with increasing iodoacetamide concentration. Iodoacetamide was added five minutes after radioactive sulfate in these experiments.

Table II

Effect of Iodoacetamide Concentration on Sulfate Incorporation

Radioautograph no.	Iodoacetamide conc (M/L)	cpm/0.1 ml cells			
		AE	EE	HE	Total recovered
		× 10^{+5}	× 10^{+5}	× 10^{+5}	× 10^{+5}
1-15	0	2.9	0.28	6.0	9.3
2-15	10^{-5}	2.8	0.28	4.5	7.6
3-15	10^{-4}	3.8	0.32	5.2	9.3
4-15	10^{-3}	5.2	0.17	4.3	9.6
5-15	10^{-2}	5.9	0.26	3.5	9.7

Each tube contained 0.1 ml volume of Chlorella cells in 1.0 ml sulfur-free medium. Each received 12 × 10^{+5} cpm $S^{35}O_4^{=}$ at zero time. Iodoacetamide was added 5 min after the radioactive sulfate. Tubes were incubated for 1 hr and extracted with HCl. Incorporation into the acid solubles (AE), ethanol solub'es (EE) and insolubles (HE) is shown.

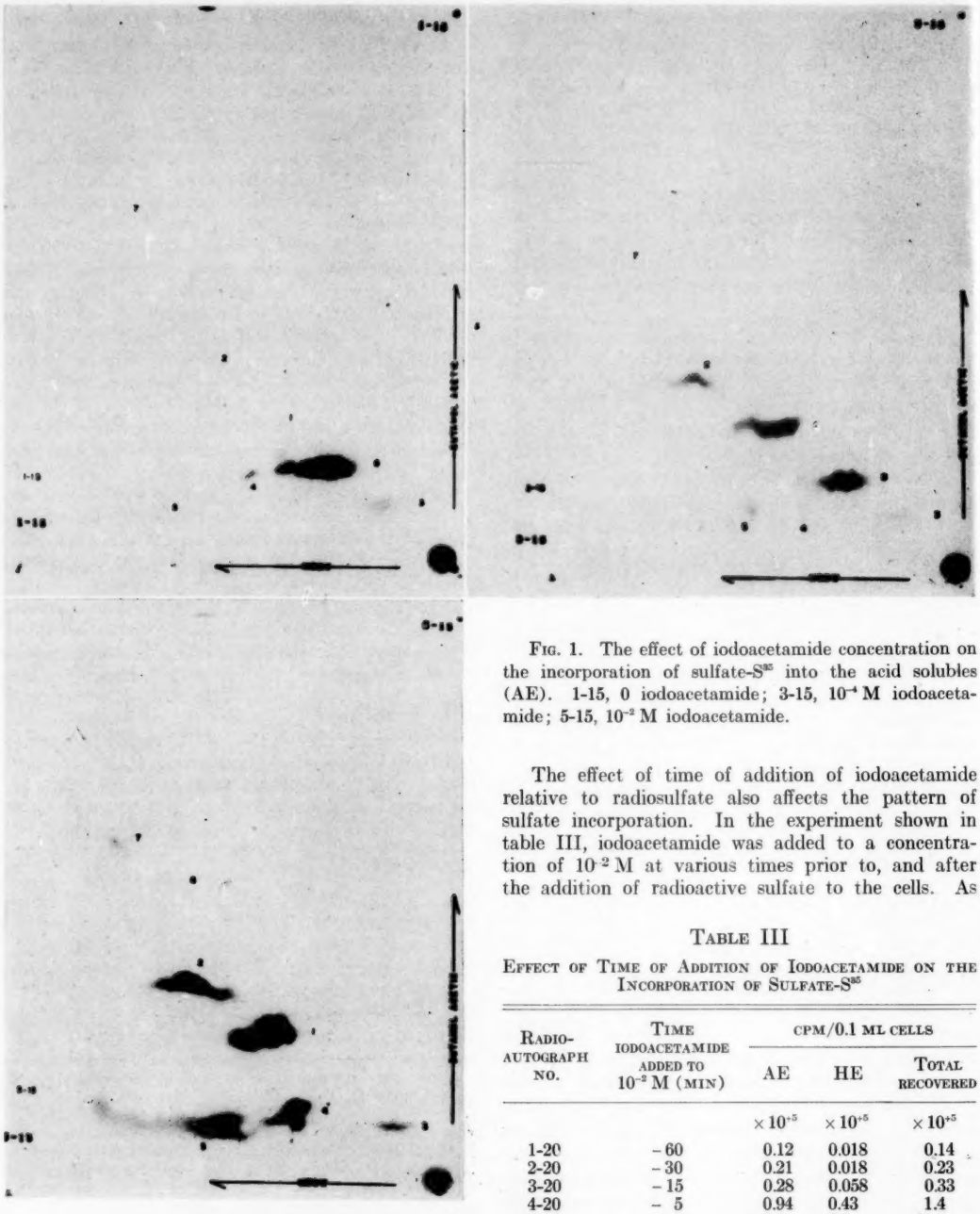


FIG. 1. The effect of iodoacetamide concentration on the incorporation of sulfate-S^{35} into the acid solubles (AE). 1-15, 0 iodoacetamide; 3-15, 10^{-4} M iodoacetamide; 5-15, 10^{-2} M iodoacetamide.

A comparison of the radioautographs of the acid extracts (AE) at various iodoacetamide concentrations shown in figure 1 reveals the disappearance of compound 8 with the concomitant appearance of several other compounds as the iodoacetamide concentration is increased.

The effect of time of addition of iodoacetamide relative to radiosulfate also affects the pattern of sulfate incorporation. In the experiment shown in table III, iodoacetamide was added to a concentration of 10^{-2} M at various times prior to, and after the addition of radioactive sulfate to the cells. As

TABLE III

EFFECT OF TIME OF ADDITION OF IODOACETAMIDE ON THE INCORPORATION OF SULFATE-S^{35}

RADIOAUTOGRAPH NO.	TIME IODOACETAMIDE ADDED TO 10^{-2} M (MIN)	CPM/0.1 ML CELLS		
		AE	HE	TOTAL RECOVERED
		$\times 10^{+5}$	$\times 10^{+5}$	$\times 10^{+5}$
1-20	−60	0.12	0.018	0.14
2-20	−30	0.21	0.018	0.23
3-20	−15	0.28	0.058	0.33
4-20	− 5	0.94	0.43	1.4
5-20	0	9.0	5.9	15.0
6-20	10	5.0	8.8	14.0
7-20	No iodoacetamide	4.1	9.1	13.0

Each tube contained 0.1 ml volume of Chlorella cells in 1.0 ml of sulfur-free medium. Each received 17×10^5 cpm $S^{35}O_4^=$ at zero time. Tubes were incubated for 1 hr after $S^{35}O_4^=$ addition. HCl was the extracting solution.

TABLE IV

INCUBATION OF CHLORELLA EXTRACT WITH RAT LIVER ENZYMES

TUBE NO.	CHLORELLA PREP	RAT ENZYMES	VERSENE 0.5 M	Mg 0.2 M	ATP 0.1 M	$SO_4^=$ 0.15 M	NITROPHENOL SULFATE FORMED *
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*μg*
1	3.0	...	...	...	...	...	17
2	3.0	1.0	...	...	...	...	56
3	3.0	1.0	0.3	...	...	...	58
4	...	1.0	...	...	...	...	0
5	...	1.0	...	0.1	0.1	0.3	80
6	...	1.0	...	...	0.1	0.3	6
7	...	1.0	0.3	0.1	0.1	0.3	2

All tubes contained 1.0 ml phosphate buffer (0.1 M, pH 7.1), 100 μg *p*-nitrophenol and enough distilled water to bring to 5.5 ml.

* The formation of nitrophenol sulfate was measured by the disappearance of the color due to *p*-nitrophenol after a correction for turbidity.

may be seen from 1-20 to 4-20 in table IV, iodoacetamide added prior to the addition of radioactive sulfate almost completely inhibited the uptake of sulfate by the cells. The simultaneous addition of iodoacetamide and radiosulfate (5-20) permitted a large uptake of the radiosulfate into both the acid solubles (AE) and the insolubles (HE) as did iodoacetamide added 10 minutes after radiosulfate (6-20).

The radioautographs of the acid solubles under these conditions are shown in figure 2. As may be seen from radioautograph 4-20 in figure 2, iodoacetamide added five minutes prior to radiosulfate has inhibited the spread of the label from sulfate to other compounds of the acid extracts. The same situation is found for the simultaneous addition of radiosulfate and iodoacetamide; in this case even though the

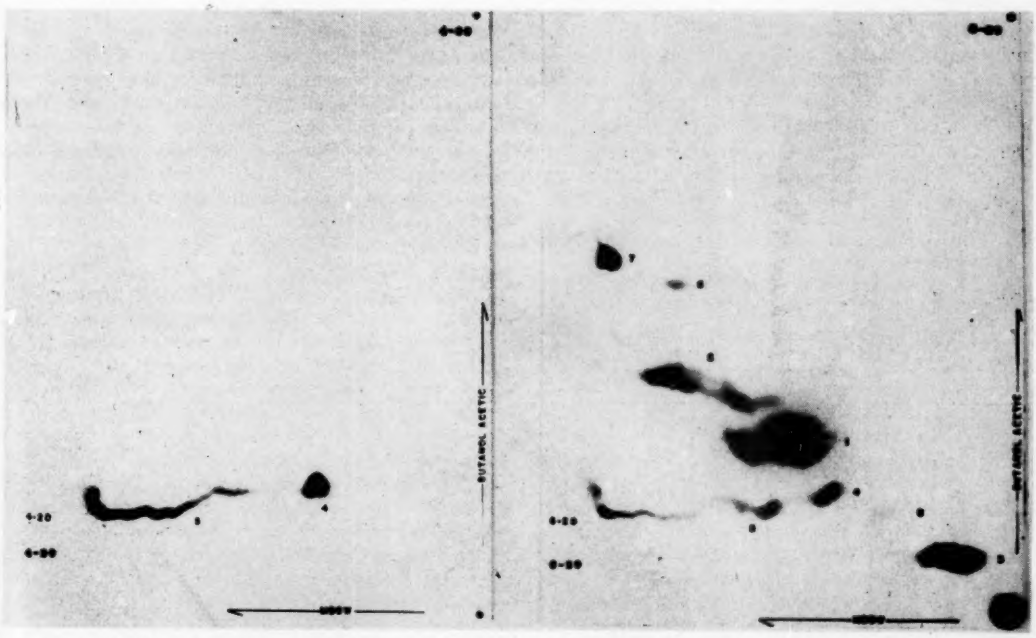


FIG. 2. The effect of time of addition of iodoacetamide relative to sulfate-S^{35} on the incorporation of sulfate-S^{35} into the acid solubles (AE). 4-20, iodoacetamide added 5 minutes prior to radiosulfate; 6-20, iodoacetamide added 10 minutes after radiosulfate.

spread of sulfate into the other compounds of the acid extracts (AE) is inhibited, there is still a considerable incorporation of label into the insolubles (HE). If iodoacetamide is added 10 minutes after the addition of radioactive sulfate, the same radioautographic pattern is found as was obtained in the previous experiment for the addition of iodoacetamide five minutes after the addition of radiosulfate. (Compare figure 1, 5-15 and figure 2, 6-20.)

The effect of iodoacetamide addition between 0 and 10 minutes after radiosulfate was explored using paper electrophoresis to separate the compounds of the acid solubles (AE). If iodoacetamide is added three minutes after the addition of radioactive sulfate, the pattern of compounds is different from that found for the addition of iodoacetamide five minutes after the isotopic compound. Since the pattern found at five minutes is the same as that found at 10 minutes, the spread of radioactivity into the acid extracts (AE) from radiosulfate is probably complete by five minutes after the addition of radiosulfate to the cells.

One of the compounds found on the three-minute electrophoretograms had a high mobility towards the anode. The possibility arose that this compound might be an "active" sulfate as described by Hilz and Lipmann (7). A cross incubation between a rat liver enzyme system in which the activating enzyme had been inactivated with versene, and an extract of Chlorella which received iodoacetamide three minutes after radiosulfate was carried out to test this possibility. The transferase in the liver extract should catalyze the reaction of *p*-nitrophenol with any "active" sulfate in the Chlorella extract to yield *p*-nitrophenol sulfate. The results of such an experiment are shown in table IV.

The tubes which contained the Chlorella preparation alone (tube 1), the rat liver enzyme alone (tube 4), the complete rat enzyme system without magnesium (tube 6), and the complete rat enzyme system with added magnesium and versene (tube 7) failed to produce a significant amount of nitrophenol sulfate. The Chlorella preparation plus the unfortified rat enzyme system (tube 2) produced a significant amount of nitrophenol sulfate, as did the same combination with added versene (tube 3). The largest formation of nitrophenol sulfate was observed in the completely fortified rat liver enzyme system (tube 5). The results suggest that a compound similar to the "active" sulfate found in rat liver is present in Chlorella during sulfate utilization. Actual proof will have to await studies on cell-free enzyme systems of Chlorella.

Identification of S-adenosyl Methionine: In attempts to identify the radioactive compounds produced from radiosulfate utilization, indirect methods of chromatography and electrophoresis were employed since the amount of compounds isolated was small. Evidence obtained from paper electrophoresis, and ion-exchange chromatography on Dowex-50 indicates that compounds of several degrees of acidity and basicity are present in the acid extracts (AE). A description of the separation of the radioactive compounds of the acid extracts on Dowex-50 is shown in table V.

Only one of the compounds present in the acid extracts has been identified with any certainty. This is compound 3 shown in figure 2. Compound 3 was suspected to be quite polar due to its low R_f in both solvent systems employed. This was confirmed by chromatography on Dowex-50 and paper electrophoresis where it behaved as a fairly strong base. If one assumed that the basicity of the molecule was due to the sulfur atom(s) which it contained then a sulfonium configuration suggested itself. When the amount of extract placed on the chromatographs was greatly increased, a quenching spot under light of approximately 260 mμ was observed to be coincident with the radioactivity from spot 3. These properties suggested that the compound might be S-adenosyl methi-

Table V

Separation of S^{35}-labeled Compounds on Dowex-50

Fraction no.	Normality of HCl used to elute	ml HCl collected per fraction	cpm in total fraction **	Compounds present *	Examples of known compounds separated by Tabachnik and Tarver (19)
			$\times 10^{+5}$		
D-1	0.0	125	1.59	2, 4, 5	Taurine, cysteic acid
D-2	1.5	112	1.24	2	.....
D-3	2.5	615	0.86	8, 1 (1)	Glutathione, methionine, methionine sulfoxide, cystine, cystathionine
D-4	4.0	150	0.08	.....	.....
D-5	4.0	180	0.06	.....	.....
D-6	6.0	190	0.27	3	.....
D-7	6.0	160	0.08	.....	.....

* The numbers refer to the spots found on subsequent paper chromatography of these fractions in MBBW, butanol-acetic. The numbers are the same as given in Figure 2.

(1) Three compounds now occupy the place on the paper formerly occupied by only one.

** CPM placed on the column at the start: 5.15×10^6; total recovered: 4.18×10^6.

onine which had been described from mammalian systems by Cantoni (2). An authentic sample of S-adenosyl methionine kindly provided by Dr. Cantoni, showed exact co-chromatography with spot 3 and a similar electrophoretic mobility. It has been shown that S-adenosyl methionine yields thiomethyladenosine on mild hydrolysis (2). Mild hydrolysis of spot 3 yielded a compound whose R_f values were similar to those of an authentic sample of thiomethyladenosine in our solvent systems. Exact co-chromatography of known compounds of biological importance, other than S-adenosyl methionine with the unknown spots has not been realized. Partial coincidence of some compounds has been observed suggesting that some of the unknown spots may contain more than one compound. For example, spot 1 partially co-chromatographs with synthetically prepared S-cysteine acetamide. It is possible that spot 1 also contains the acetamide derivatives of closely related sulfhydryl compounds such as homocysteine or glutathione. It is hoped that in the future the application of a combination of techniques of separation will permit the further identification of these compounds.

Discussion and Conclusions

We have attempted to establish conditions which would lead to control of the rapid process of sulfate utilization and which would yield reproducible chromatographic patterns of compounds that are formed from sulfate. This has been realized through the employment of relatively mild extraction techniques and by the use of iodoacetamide as an inhibitor of the sulfate reduction process. It was found that the simultaneous addition of iodoacetamide and radiosulfate to the cells results in a large uptake of label but the radioactivity found in the acid extracts under these conditions remains largely as sulfate. If iodoacetamide is added prior to radiosulfate, the uptake of label is strongly inhibited. This evidence suggests that at least one site of iodoacetamide inhibition occurs very early in the metabolism of sulfate; perhaps the site of inhibition may be the activating system itself. The activity of the sulfate activating system of mammalian tissues is inhibited by O-[(3 hydroxymercuri-2-methoxypropyl)] carbamyl phenoxyacetic acid; this inhibition is reversed by cysteine (7). Studies of our own indicate that the sulfate activating system of mammalian systems is sensitive to iodoacetamide (Whitehouse, M. and Schiff, unpublished). It is possible, therefore, that the sulfate activating system of Chlorella is sensitive to iodoacetamide and that this system is the site of inhibition observed in the experiments described above. An equally likely possibility to be considered is that iodoacetamide is merely inhibiting the systems of energy production within the cell which is thereby deprived of an energy source for active accumulation of sulfate.

Although the simultaneous addition of radiosulfate and iodoacetamide inhibits the spread of label into other compounds of the acid extracts, considerable label is still incorporated into the insoluble fraction which has not yet been fractionated. The label in this fraction may reside only in sulfate conjugates of polysaccharides and other large molecules. If the label should be present in reduced form such as the amino acids of protein, however, this would indicate that there may be a route of sulfate reduction in Chlorella which does not involve the compounds of the acid extracts.

The mechanism by which S-adenosyl methionine is formed in Chlorella may be different from the activating system described by Cantoni from mammalian systems (2). In our experiments reported previously (21) exogenous methionine was not utilized appreciably by Chlorella to form compounds of the acid extracts. The experiments described in this paper show that adenosyl methionine is formed during the reduction of sulfate by this organism. It would be tempting to visualize S-adenosyl methionine as one of the last compounds in a chain beginning with "active sulfate" and leading, perhaps, through a series of sulfur conjugates of AMP to more reduced sulfur compounds. Bandurski has recently reported a system which is capable of releasing pyrophosphate from ATP in the presence of inorganic sulfite (30). The compound of adenylic acid and sulfite which one would expect has proven to be too unstable to be isolated. Although this compound is very unstable, it could conceivably play a part in such a chain of reduction products of sulfate. It is also interesting to note that the sulfate activating system has been found in yeast and in Neurospora both of which are capable of reducing inorganic sulfate. The sulfate activating system in mammalian cells, which are incapable of reducing sulfate, is accompanied by a phenol transferase system while the phenol transferase system is apparently absent from Neurospora which is capable of carrying out the reduction process.

Although many spots corresponding to compounds formed from radioactive sulfate are found on the reported chromatograms, the only compound which we have identified with any certainty is S-adenosyl methionine. It is hoped that the other compounds found will be identifiable in future studies and that the elucidation of their role in the intermediary metabolism of sulfate will similarly be possible.

Summary

Sulfate-S^{35} is taken up rapidly by *Chlorella pyrenoidosa* and is converted to reduced compounds within the cell. One of the compounds formed from $S^{35}O_4^=$ shows the properties of S-adenosyl methionine. Preliminary evidence is also presented for the formation of "active" sulfate. Techniques have been developed to stabilize and control the process of sulfate reduction by Chlorella by using iodoacetamide as an inhibitor. These techniques are described.

The author would like to thank Professor William Stepka, Professor David R. Goddard and Professor Martin Gibbs for their encouragement throughout the

course of this work. He would like to thank Dr. Michael Whitehouse for the rat liver enzyme used in these studies, Dr. Giulio Cantoni for a generous supply of S-adenosyl methionine, and Dr. F. Schlenk for a sample of thiomethyladenosine given to Professor Stepka. He would also like to thank Professor Samuel Gurin and Dr. Whitehouse for many stimulating discussions of sulfur biochemistry.

Literature Cited

1. Bandurski, R. S., Wilson, L. G. and Squires, C. L. The mechanism of "active sulfate" formation. Jour. Amer. Chem. Soc. 78: 6408–6409. 1956.
2. Cantoni, G. L. S-adenosylmethionine, a new intermediate formed enzymatically from L-methionine and adenosinetriphosphate. Jour. Biol. Chem. 204: 403–416. 1953.
3. Cantoni, G. L. and Durell, J. Methionine activating enzyme. Federation Proc. 15: 299. 1956.
4. Cowie, D. B., Bolton, E. T. and Sands, M. K. Competitive utilization of labeled and nonlabeled sulfur compounds. Jour. Bact. 62: 63–74. 1951.
5. deMeio, R. H., Wizerkaniuk, M. and Schreibman, I. Enzymatic system synthesizing sulfuric acid esters of phenols. Jour. Biol. Chem. 213: 439–443. 1955.
6. Gibbs, M. and Schiff, J. A. Chemosynthesis, energy relations in autotrophic organisms. (In preparation.)
7. Hilz, H. and Lipmann, F. The enzymatic activation of sulfate. Proc. Natl. Acad. Sci., U.S. 41: 880–890. 1955.
8. Hopkins, E. F. Iron-ion concentration in relation to growth and other biological proceses. Bot. Gaz. 89: 209–240. 1930.
9. Horowitz, N. H. Methionine synthesis in Neurospora. The isolation of cystathione. Jour. Biol. Chem. 171: 255–264. 1947.
10. Horowitz, N. H., Fling, M., Phinney, B. O. and Shen, S. Recent experiments on the methionine-requiring mutants of Neurospora. Abstracts of San Francisco meeting of the Amer. Chem. Soc., p. 45c. 1949.
11. Horowitz, N. H. In: Symposium on Amino Acid Metabolism. Pp. 631–632. Johns Hopkins Press, Baltimore 1955.
12. Lampen, J. O., Roepke, R. R. and Jones, M. J. Studies on the sulfur metabolism of *E. coli*. III. Mutant strains of *E. coli* unable to utilize sulfate for their complete sulfur requirements. Arch. Biochem. Biophys. 13: 55–66. 1947.
13. Latimer, W. M. Oxidation Reduction Potentials. Pp. 65–66. Prentice-Hall, New York 1938.
14. Liverman, J. L. and Ragland, J. B. Metabolism of sulfur-35 in the Alaska pea. Plant Physiol. 31 Suppl.: vii. 1956.
15. Mason, M. and Berg, C. P. A chromatographic method for the detection of tryptophane metabolites. Jour. Biol. Chem. 188: 783–788. 1951.
16. McRorie, R. A. and Carlson, G. L. Methionine precursors in Neurospora. Federation Proc. 15: 313. 1956.
17. Phinney, B. O. Cysteine mutants in Neurospora. Genetics 33: 624. 1948.
18. Ragland, J. B. and Liverman, J. L. A reinvestigation of the sulfur auxotrophs of Neurospora. Plant Physiol. 31 Suppl.: viii. 1956.
19. Robbins, P. W. and Lipmann, F. Identification of enzymatically active sulfate as adenosine-3′-phosphate-5′-phosphosulfate. Jour. Amer. Chem. Soc. 78: 2652–2653. 1956.
20. Robbins, P. W. and Lipmann, F. The enzymatic sequence in the biosynthesis of active sulfate. Jour. Amer. Chem. Soc. 78: 6409–6418. 1956.
21. Schiff, J. A. Preliminary studies on the sulfur metabolism of *Chlorella pyrenoidosa* with sulfur-35. Ph.D. thesis, University of Pennsylvania. Dissertation Abstract No. 17, 271. 1956.
22. Schiff, J. A. Preliminary studies on the sulfur metabolism of *Chlorella pyrenoidosa* with sulfur-35. Plant Physiol. 31 Suppl.: vii. 1956.
23. Schlenk, F. and Smith, R. J. The mechanism of adenine thiomethylriboside formation. Jour. Biol. Chem. 204: 27–34. 1953.
24. Schwartz, M. and Shapiro, S. K. The mechanism of utilization of thiomethyladenosine in the biosynthesis of methionine. Jour. Bact. 67: 98–102. 1954.
25. Singer, T. P. and Kearney, E. B. In: Symposium on Amino Acid Metabolism. Pp. 587–588. Johns Hopkins Press, Baltimore 1955.
26. Stepka, W. In: Methods in Medical Research, A. C. Corcoran, ed. Vol. 5. Pp. 25–62. New Year Book Publishers, Chicago 1952.
27. Tabachnick, M. and Tarver, H. The conversion of methionine-S^{35} to cystathione-S^{35} and taurine-S^{35} in the rat. Arch. Biochem. Biophys. 56: 115–122. 1955.
28. Teas, H. J., Horowitz, N. H. and Fling, M. Homoserine as a precursor of threonine and methionine in Neurospora. Jour. Biol. Chem. 172: 651–658. 1948.
29. von Braun, J. Über die Doppeldissoziation Quartärer Ammoniumverbindungen und eine Bequeme Synthese des Jod-acetonitrils. Ber. deut. chem. Ges. 41: 2130–2144. 1908.
30. Wilson, L. G. and Bandurski, R. S. An ATP-sulfite reaction. Plant Physiol. 31 Suppl.: viii. 1956.
31. Zweig, G. and Hood, S. L. Sulfate fixation by *Chlorella pyrenoidosa*. Abstracts of Dallas Meetings of the Amer. Chem. Soc. Abstract No. 66. 1956.

ELECTRON TRANSPORT MECHANISMS IN TOBACCO ROOTS. I. STUDIES ON THE CYTOCHROME AND RELATED SYSTEMS[1,2]

EDWARD C. SISLER[3,4] AND HAROLD J. EVANS

DEPARTMENT OF BOTANY, NORTH CAROLINA STATE COLLEGE, RALEIGH, NORTH CAROLINA

Since the work of Keilin (16), several investigators have made extensive studies on the distribution and properties of cytochrome enzymes in various higher plant materials (1, 19, 20, 30). Some attempts have been made to purify cytochromes from higher plants. Goddard (7) has purified cytochrome c from wheat germ and Hill and Scarisbrick (12) have purified cytochromes b_3 and c from bean leaves. Miller et al (22) have dispersed cytochrome oxidase from soybeans using sodium cholate, but no appreciable purification was accomplished. Simon (23) observed that treatment of plant mitochondria with digitonin greatly increased the cytochrome c oxidative capacity and he suggests that dispersion by digitonin increases the accessibility of cytochrome oxidase to exogenous reduced cytochrome c. No purification of cytochrome oxidase from the digitonin extracts was reported.

Hackett (8) has studied electron transport enzymes in particles isolated from potatoes and has identified cytochrome oxidase, DPNH (reduced diphosphopyridine nucleotide) oxidase, diaphorase, DPNH cytochrome c reductase and succinic cytochrome c reductase. He suggests with the particulate system that electrons are transferred successively from DPNH to a flavoprotein, to an antimycin A sensitive factor, to the cytochrome system and then to oxygen. In a study of the properties of a soluble preparation from potato tissue Hackett (9) has demonstrated that extracts prepared in presence of cysteine will catalyze the oxidation of either DPNH or TPNH (reduced triphosphopyridine nucleotide) by molecular oxygen. Dialysis of the extracts caused a loss of activity. Humphreys and Conn (15) have studied the properties of a system in lupine mitochondria which catalyzed the oxidation of reduced pyridine nucleotide by molecular oxygen.

There is relatively little evidence to indicate the significance of the cytochromes in the tobacco plant. Cytochrome oxidase has been identified in extracts of the tobacco plant by Webster (30) and by Miller and Evans (21) and a pyridine nucleotide-cytochrome c reductase has been identified in extracts of tobacco leaves (5). Consequently, the purpose of this investigation was to obtain more detailed information on the occurrence and relative significance of the cytochrome and closely related electron transport enzymes in tobacco roots.

MATERIALS AND METHODS

EXTRACTION OF THE ENZYMES: Tobacco plants (*Nicotiana Tabacum* L. var. Dixie Bright 101) were grown in solution culture in the greenhouse. When the plants were nearly mature the roots were removed and new initials allowed to develop. Young roots ranging from 7 to 10 days in age were ground with sand in a chilled mortar with a volume of medium in milliliters equal to the weight in grams of the roots. The homogenizing medium contained 0.5 M sucrose, 0.1 M potassium phosphate buffer at pH 7.0 and 10^{-3} EDTA (disodium salt of ethylenediaminetetraacetic acid). The resulting homogenate was strained through cheese cloth and the liquid extract centrifuged at $500 \times G$ for 5 minutes. The supernatant liquid (crude extract referred to in table I) was decanted

TABLE I

COMPARISON OF SODIUM CHOLATE AND DIGITONIN FOR THE DISPERSION OF CYTOCHROME OXIDASE FROM TOBACCO ROOTS

PREPARATION *	VOLUME	TOTAL ACTIVITY	PROTEIN CONTENT	SPECIFIC ACTIVITY
	ml	*units*	*mg/ml*	*units/mg protein*
I Crude extract	250	1,160,000	5.6	840
II Digitonin extract of 50 % of the particles from I	30	940,000	1.4	22,000
III Cholate extract of 50 % of the particles from I	30	240,000	3.0	2,600

* The crude extract was divided into 2 equal parts. The particles from one part were extracted with 2 % sodium cholate and the particles from the other were extracted with 2 % digitonin. In this experiment 125 grams of roots were used for the preparation of the extracts. Details of the preparation and assay are described under Materials and Methods. No reaction occurred in the absence of enzyme or in the presence of enzyme held at a boiling temperature for 1 minute.

[1] Received revised manuscript August 15, 1958.

[2] Contribution from the Department of Botany, North Carolina Agricultural Experiment Station and published with the approval of the Director as paper No. 877 of the Journal Series.

[3] The data reported here are taken from the Ph.D. thesis of Edward C. Sisler, presented to the Graduate School of the North Carolina State College, June, 1958.

[4] Present address: Biology Department, Brookhaven National Laboratory, Upton, N. Y.

and the sediment discarded. The supernatant liquid was centrifuged at 25,000 × G for 20 minutes and the sediment of particles was collected and utilized for various preparations as indicated.

When particulate material was desired the 25,000 × G sediment was suspended in a sufficient volume of the sucrose-phosphate-EDTA medium to obtain the desired enzyme activity. When a dispersed preparation was desired, about 5 ml of packed particles were suspended in 15 ml of 0.1 M phosphate buffer at pH 7.0 containing 10^{-3} M EDTA. A sufficient quantity of either digitonin or sodium cholate and water was added to give a final concentration of 2 % with respect to the detergent. The total volume was increased to 30 ml by this procedure. After approximately one hour in contact with the detergent, the preparation was centrifuged at 25,000 × G for 20 minutes. The sediment was discarded and the supernatant saved for assay or further purification. All of these operations were carried out between 0 and 4° C.

Chemicals: The digitonin or cholic acid was dissolved in a small amount of 10 % NaOH. After adjusting the pH to 8.5 with HCl the solutions were diluted to the correct volume for the desired concentration. DPNH (diphosphopyridine nucleotide), 90 % purity) and cytochrome c (0.34 % iron) were obtained from the Sigma Chemical Company, St. Louis, Missouri. The concentration of DPNH was determined with a Beckman DU spectrophotometer assuming the extinction coefficient of 6.24×10^6 cm^2 × moles^{-1} at 340 mμ (14). The concentration of cytochrome c was determined with the same instrument at 550 mμ using the difference in molar extinction coefficients of the oxidized and reduced forms which is 1.96×10^4 (13). The cytochrome c was reduced with sodium hydrosulfite and aerated for a few minutes to destroy any excess sodium hydrosulfite (4). The Antimycin A was obtained from the Wisconsin Alumni Research Foundation, Madison, Wisconsin, and dissolved in 95 % ethanol. The SN 5949 [2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone generously supplied by Dr. James Hilton] was dissolved in 1 % NaOH, the pH adjusted to 8.5 with HCl and then diluted to the desired concentration.

Assay Procedures: The standard assay procedure for determination of cytochrome c oxidase activity (4) involved the measurement of the rate of the decrease in optical density of reduced cytochrome c at 550 mμ by use of a Beckman DU spectrophotometer. The assay mixture in a final volume of 1 ml contained 0.1 M potassium phosphate buffer at pH 7.0, 10^{-3} M EDTA, 5.2×10^{-5} M cytochrome c and the enzyme extract. One unit of activity is defined as the amount of enzyme that will cause an optical density change of 0.001 during the 15- to 75-second interval of time after the reaction began.

As a control reaction for each of the cytochrome oxidase assays, all constituents of each reaction mixture except the cytochrome oxidase preparations were added to the cuvette and the rate of increase in absorbence at 550 mμ was determined before the enzyme was added. Also each enzyme extract was held at the temperature of boiling water for one minute and the assays were conducted with the boiled extract. Rates of decrease in absorbence when the enzyme was omitted from the reaction mixtures or rates obtained when boiled extracts were substituted for the active extracts were no greater than the normal fluctuations of the instrument. The decreases in the rate of absorbence, therefore, are due to enzyme catalysis and not due to non-enzymatic oxidation of cytochrome c.

The standard assay procedure for the determination of pyridine nucleotide cytochrome c reductase activity involved the measurement of the increase in optical density at 550 mμ (13) by use of a Beckman DU spectrophotometer. The assay mixture in a final volume of 1 ml contained 0.1 M potassium phosphate buffer at pH 7.0, 10^{-3} M EDTA, 5.2×10^{-5} M cytochrome c, 10^{-3} M KCN, the enzyme extract, and 1×10^{-4} M DPNH. Succinic-cytochrome c reductase activity was determined by the same procedure with the exception that 1.5×10^{-2} M succinate was substituted for DPNH. No measurable decrease in optical density was obtained with either determination when the extract was omitted or when boiled extract was substituted for active extract.

DPNH oxidase activity was measured by the decrease in optical density at 340 mμ. The reaction mixture in a final volume of 1 ml contained 0.1 M potassium phosphate buffer at pH 7.0, 10^{-3} M EDTA, 1×10^{-4} M DPNH and the enzyme extract. No DPNH oxidase activity was observed in the presence of boiled extracts or in the absence of active extract.

Protein contents of extracts were determined colorimetrically by the method of Lowry et al (18) using casein as a standard.

Direct Spectroscopic Methods: The absorption bands of cytochrome were determined with a Zeiss spectroscope mounted on a microscope. The apparatus was essentially the same as the Pupillen-Spectroskop described by Hartree (10). Usually about 50 g of roots were required in order to obtain a sufficient quantity of particulate material to observe the cytochrome bands. Ascorbic acid or sodium hydrosulfite at 0.03 M concentration was used during the isolation procedure in certain cases to inhibit browning. For the observation 1.5 ml of packed particles were placed in a 5-ml beaker and mixed with sufficient water to obtain a thick suspension. Sodium hydrosulfite (10) was added to reduce the cytochrome components.

Manometric Studies: Roots were harvested from solution cultures, blotted between strips of filter paper, cut into segments about 3 cm long, and weighed. Samples of roots, consisting of 0.5 g each, were placed in a Warburg flask which contained 3 ml of a complete nutrient solution in the main compartment and 0.2 ml of 20 % KOH in the center well. The manometric measurements, including the preparation of gas mixtures were carried out in accordance with the procedures outlined by Umbreit et al (28).

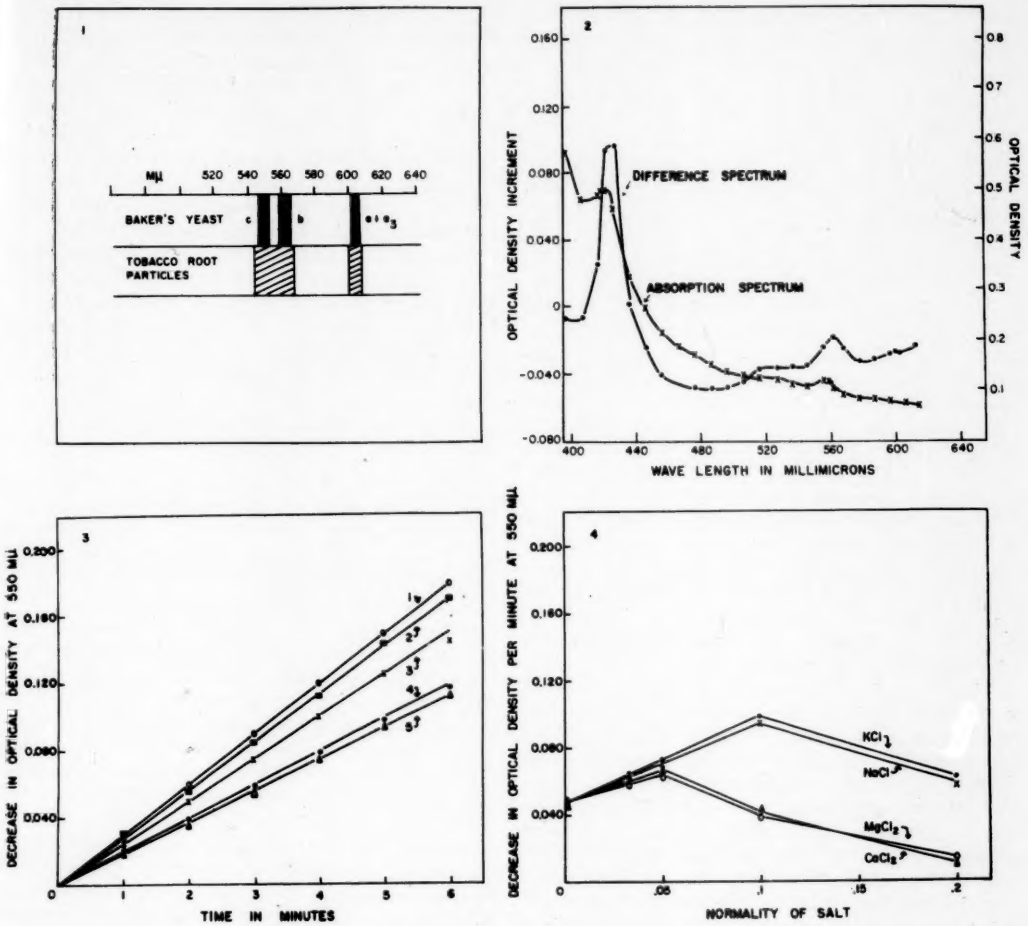


FIG. 1. The absorption bands of reduced cytochromes in baker's yeast and in tobacco root particulates. This figure indicates the position of the bands; however, the relative intensities are not indicated. The procedure is described under Materials and Methods.

FIG. 2. Difference and absorption spectra of a digitonin extract (1 mg protein per ml) of tobacco roots. The optical density increment scale applies to the difference spectrum and the optical density scale applies to the absorption spectrum. See the text for details of the experiment.

FIG. 3. Reaction kinetics of cytochrome oxidase in tobacco root extracts prepared by various methods. The protein contents were: 1.66 μg of a digitonin extract (curve 1), 25 μg of a 30 to 65% $(NH_4)_2SO_4$ precipitate of a sodium cholate extract (curve 2), 9.8 μg of an unfractionated sodium cholate extract (curve 3), 13 μg of a suspension of particulate material (curve 4), and 6.7 μg of a 0 to 30% $(NH_4)_2SO_4$ precipitate of a sodium cholate extract (curve 5). The standard assay procedure was used. All of the preparations were inactivated by holding them in a boiling water bath for 1 minute.

FIG. 4. The effect of salt concentration on cytochrome oxidase activity in a digitonin extract from tobacco roots. The standard assay procedure was used with the exceptions that 10^{-3} M TRIS buffer at pH 7.0 was included in reaction mixtures instead of phosphate buffer and the concentrations of salts were varied as indicated. No measurable cytochrome c oxidation occurred in reaction mixtures lacking extract or in mixtures containing heat inactivated extract in place of active extract.

Results

Spectroscopic and Spectrophotometric Observations: Direct observations of the particulate material with the hand spectroscope revealed two distinct bands when sodium hydrosulfite was added (fig 1). A band extending from 548 to 567 mμ was observed. The published values for the bands of cytochromes b and c fall within this range (11). The particles were treated with glycerol and subjected to the temperature of liquid nitrogen (10) but this procedure failed to resolve the 548 to 567 band into two components. This may have been due to the fact that the particles turned brown as a result of polyphenol oxidase activity, and therefore resolution was complicated. The development of the brown color in particles was decreased but not completely prevented by reducing agents such as glutathione and ascorbate. The spectroscopic assembly was satisfactory as evidenced by the fact that distinct cytochrome bands could be observed in yeast preparations and in acetone powders of leaves.

A second band near 605 mμ was visible in most preparations; however, its intensity was very low. The position of this band corresponds to published values for cytochrome a and a_3. The position of the bands in tobacco root particles are shown in figure 1, along with the bands visible in Baker's yeast, but the relative intensities are not indicated.

The absorption spectrum of an extract dispersed by the use of digitonin and reduced with sodium hydrosulfite is shown in figure 2 (see legend). The values recorded for the absorption spectrum curve are the optical densities, determined with a Beckman DU spectrophotometer when 0.1 M phosphate buffer was used in the reference cuvette. A peak is apparent at 424 mμ, and a broad band extending from 550 to 565 mμ also is shown. It is reported (11) that the peaks of the γ absorption bands of cytochrome b and c are at 432 and 415 mμ respectively. No trace of a band at 605 mμ is visible in the direct absorption spectrum.

The difference spectrum indicated in figure 2 was obtained by placing the oxidized enzyme preparation in the reference cuvette and comparing it with an equal amount of enzyme reduced by addition of sodium hydrosulfite. A peak at 428 mμ is revealed in the difference spectrum, and it is presumed that this peak is the result of the combined Soret bands of the several cytochromes present. A very faint peak near 600 mμ indicates cytochrome oxidase is present. The rather distinct peak at 562 mμ corresponds closely to published values for cytochrome b (19).

Dispersed Cytochrome Oxidase Preparations: Several methods of purification of the enzyme were attempted. Removal of the lipid material with ether or with acetone before or after extraction with detergents resulted in inactivation of the enzyme in every instance. Dialysis of a digitonin dispersed extract for three hours against 5 % butanol resulted in inactivation. Dispersing the enzyme extracts with either digitonin or sodium cholate resulted in some purification (table I). In general, more activity was obtained in extracts dispersed with digitonin than was obtained in those dispersed with cholate. Presumably this was due to the inhibitory action of sodium cholate which has been reported previously (26). As indicated in table I, the total enzyme activity was greater after the digitonin treatment than before. A comparison of various dispersing agents for the preparation of cytochrome oxidase from animal sources has been carried out by Wainio and Aronoff (29). Presumably the dispersing action of the digitonin increased the amount of enzyme exposed to the substrate. The cholate extract was fractionated by use of $(NH_4)_2SO_4$, but none of the fractions obtained were as active as the particulate extract which had been dispersed by digitonin. Much of the activity remained in solution when the digitonin extract was saturated with $(NH_4)_2SO_4$, therefore, purification by this procedure was not practical.

Reaction Kinetics of the Various Cytochrome Oxidase Preparations: Smith and Conrad (27) reported first order reaction kinetics for cytochrome oxidase in a heart-muscle extract and Miller et al (22) have reported that cytochrome oxidase activity in a 30 to 65 % $(NH_4)_2SO_4$ precipitate of cholate extracts of soybean roots decreased raidly with time as a result of enzyme inactivation. The time course of the reaction catalyzed by a particulate preparation from tobacco roots is illustrated in curve 4 of figure 3. Comparable curves of reactions catalyzed by several dispersed preparations also are shown in this figure. The amount of enzyme for each preparation was adjusted so that the amount of activity was approximately the same for the various extracts. This resulted in a wide variation in the amount of protein in each reaction mixture. All of the reaction rates remained essentially linear for several minutes at the substrate concentration used. Addition of reduced cytochrome c after the reaction stopped resulted in further cytochrome c reduction, indicating that all of these preparations differed kinetically from the 30 to 65 % $(NH_4)_2SO_4$ fraction of a sodium cholate extract from soybean roots reported by Miller et al (22). All of the preparations were completely inhibited by 10^{-3} M KCN and were inactivated by holding in a boiling water bath for one minute.

Effect of Salts on Cytochrome Oxidase Activity in a Digitonin Extract: Miller and Evans (21) have shown that salts strikingly stimulated cytochrome oxidase activity in particulates from tobacco roots and other plant materials. In another report (22) it was indicated that cytochrome oxidase activity in cholate dispersed extracts from soybean roots were not stimulated by salts. The effect of salts on the digitonin extract from tobacco roots is shown in figure 4. The shapes of the response curves are virtually the same as those reported for particulate material. Centrifugation of the digitonin preparation at an average force of 65,000 × G for one half hour resulted in no loss of activity. The activity of the

enzyme in this extract, however, still responded to salts.

OXIDATIVE CAPACITY OF TOBACCO ROOTS: An effort was made to determine whether or not tobacco roots contained sufficient cytochrome c oxidase to account for their rates of oxygen uptake. For this experiment 1-g samples were collected at random from the entire root system of vigorously growing tobacco plants of approximately 12 inches in height, and also from the roots of senescent plants (approximately 3 weeks after flowering began). Oxygen uptake was measured on both samples of excised roots by the Warburg manometric procedure. Other samples were collected from both vigorously growing and senescent plants for the determination of cytochrome c oxidative capacity. For this purpose 5-g samples of roots were ground in a mortar with 20 ml of a solution containing 0.1 M potassium phosphate buffer at pH 7.0, 10^{-3} M EDTA and 2 % digitonin. After standing for one hour the preparations were centrifuged at $500 \times G$ for 10 minutes. This procedure extracted most of the cytochrome oxidase from the insoluble material. The cytochrome oxidase activities of the extracts were determined by the spectrophotometric assay previously described.

TABLE II

ELECTRON TRANSPORT CAPACITY OF VARIOUS SYSTEMS IN YOUNG TOBACCO ROOTS

DETERMINATION	MATERIALS ASSAYED	ACTIVITY/G OF ROOTS/HR *
		electron equivalents
Oxygen uptake	Root segments	4.0×10^{-5}
Cytochrome c oxidized	Crude extract **	4.6×10^{-5}
Cytochrome c oxidized	Digitonin extract **	3.0×10^{-4}
Cytochrome c reduced	Crude extract	4.6×10^{-5}
Cytochrome c reduced	Digitonin extract	1.8×10^{-5}
Cytochrome c reduced	Crude extract $+ 2 \times 10^{-5}$ M menadione	$1.2 \times 1\,0^{-3}$
Cytochrome c reduced	Digitonin extract $+ 2 \times 10^{-5}$ M menadione	1.2×10^{-3}

* Oxygen uptake was determined by the manometric procedure, and cytochrome oxidative and reductive capacities were determined spectrophotometrically by the standard procedures for these enzymes. These determinations were carried out at 25° C. No measurable reaction occurred in the absence of enzyme or in the presence of enzyme previously held at a boiling temperature for 1 minute.

** Details of the preparation of the crude extract are described under Materials and Methods. The preparation of the digitonin extract used for determination of both cytochrome oxidative and reductive capacities is described in the text under Oxidative Capacity of Tobacco Roots.

The results of this experiment, tabulated in table II, indicate that the digitonin extracts of roots of young vigorously growing plants contained about 7.5-fold greater activity of cytochrome oxidase than would be required to account for the rate of oxygen uptake by intact roots. The cytochrome oxidase activity in the extract from roots of senescent plants (not tabulated) is about four-fold that required to account for the oxygen uptake by the intact roots. The total cytochrome oxidase activity of crude extracts of young roots prepared as described under "Materials and Methods" approximately equaled the oxygen uptake in electron equivalents.

K_M OF CYTOCHROME OXIDASE FOR OXYGEN: Since an objective of this study was to determine the relative significance of the cytochrome systems in the metabolism of tobacco roots, it was deemed necessary to estimate the Michaelis constant of cytochrome oxidase for oxygen. This constant may be useful in evaluating the significance of cytochrome oxidase in relation to other terminal oxidases found in tobacco roots. Since the concentration of oxygen necessary for saturation of cytochrome oxidase is exceedingly low, it was not feasible to prepare gas mixtures for this experiment. The following procedure was used therefore for estimation of K_M for oxygen.

A reaction mixture in a final volume of 3 ml, containing 0.1 M phosphate buffer at pH 7.0, 10^{-3} M EDTA, 1.02×10^{-4} M reduced cytochrome c and 0.02 ml of digitonin extract of roots (1.4 mg protein per ml) was placed in a cuvette with a Thunberg attachment. As illustrated in figure 5 the reaction was linear with time over the optical density change occurring during the reaction, indicating that the enzyme was saturated with respect to reduced cytochrome c and also with oxygen when the cuvette was open to the atmosphere. A comparable reaction mixture was prepared with the enzyme in the side arm of the Thunberg cuvette and the other constituents in the main compartment, and then the cuvette was evacuated and filled with oxygen free argon. The evacuation and admission of argon was repeated several times shaking the cuvette before and after each step of the procedure. Since it was desired to limit the oxygen concentration to a point that would allow several spectrophotometric readings before the reaction was halted by lack of oxygen, several reaction mixtures were evacuated by trial and error until the desired reaction rate was obtained. The reaction, indicated by the curve in figure 5 for the partially evacuated cuvette, was started by tipping the enzyme into the main compartment. Optical densities were recorded for each 15-second interval for 6 minutes. After a total reaction time of 8 minutes, the reaction had reached a very definite equilibrium. The optical density was observed up to 21 minutes and no further change was noted. At this point it was assumed, for this determination, that the oxygen concentration was zero. No endogenous reduction of cytochrome c could be observed in these preparations when 10^{-3} M KCN was present in reaction mixtures.

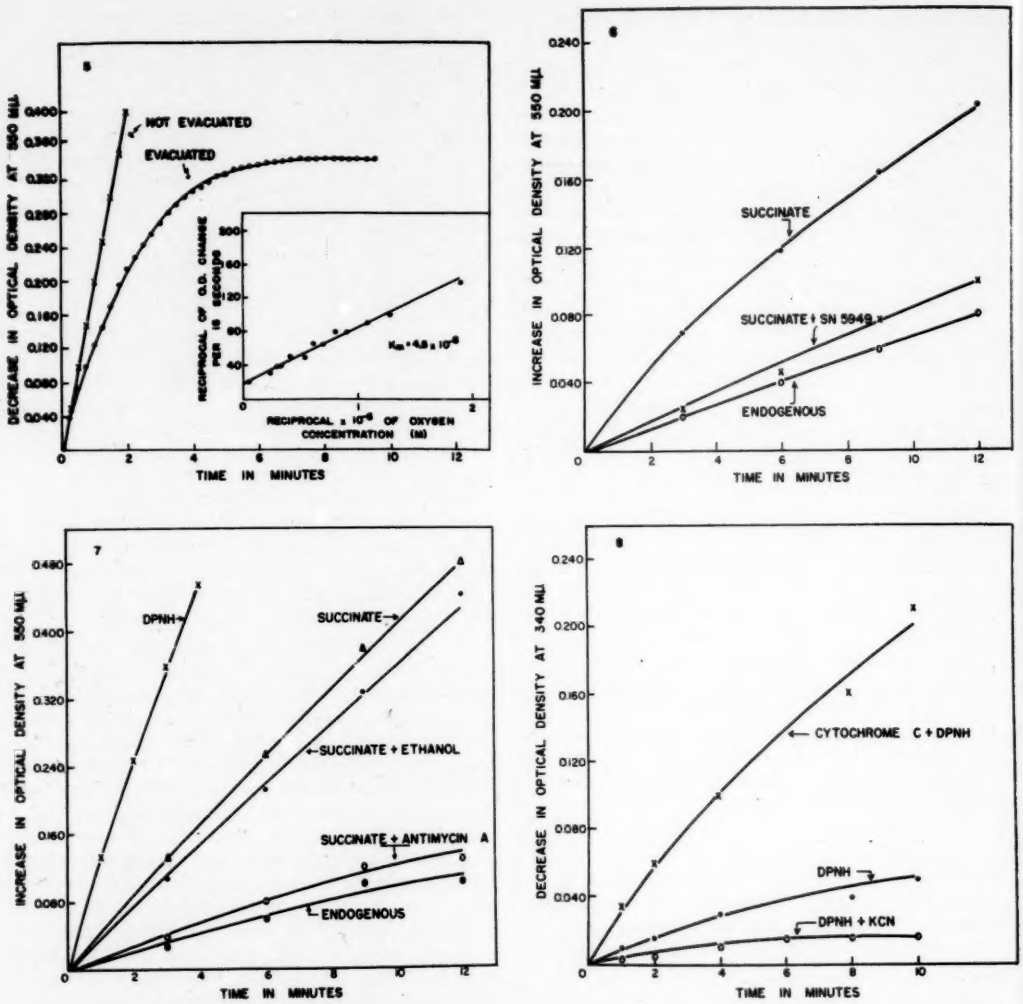


FIG. 5. (Major graph) The effect of oxygen concentration on the reaction kinetics of cytochrome oxidase. The linear curve represents the time course of the reaction when the cuvette was not evacuated and therefore was in equilibrium with the atmosphere. The lower curve indicates the time course of the reaction when the cuvette was evacuated sufficiently to limit the oxygen supply. No measurable reaction occurred before tipping the enzyme into the main compartment of the cuvette.

(Inset) A recpirocal plot of reaction velocity (ordinate) and molar oxygen concentration (abscissa). The data were calculated from the curvilinear reaction rate of the evacuated reaction (Major graph). See the text for details of the experiment.

FIG. 6. Succinic-cytochrome reductase activity of tobacco root particulates and its inhibition by SN 5949. The standard assay procedure was utilized with the exceptions that 3 μg of SN 5949 were included as indicated and succinate was omitted in the endogenous reaction. The particulate extract contained 0.64 mg of protein.

FIG. 7. A comparison of DPNH- and succinic-cytochrome c reductase activities of tobacco root particulates, and the effect of Antimycin A (1 μg) on succinic-cytochrome c reductase. The standard assay procedures were utilized. When succinic-cytochrome c reductase activity was determined a quantity of extract was added containing 0.8 mg of protein. When DPNH-cytochrome c reductase was measured only 0.06 mg protein from the same extract was added.

FIG. 8. The oxidation of DPNH by tobacco root particles, and its activation by cytochrome c and inhibition by KCN. The standard assay procedure for DPNH oxidase was used with the exceptions that cytochrome c at a concentration of 5.2×10^{-6} M and KCN at a concentration of 10^{-3} M were added as indicated. The extract in each reaction contained 0.06 mg protein.

Using the difference in extinction coefficients of oxidized and reduced cytochrome c at 550 mμ, the molar concentration of cytochrome c that was oxidized at each point on the curve was calculated. Since four electrons are required for the reduction of one molecule of oxygen, the molarity of the oxygen consumed also was calculated assuming the oxygen concentration to be zero at the point of equilibrium. It could be demonstrated that approximately 85 % of the cytochrome c remained reduced at equilibrium and at this point admission of air caused the reaction to proceed. The velocity of the reaction at each point on the curve was estimated by the slope of a line drawn tangent to each point. These rates and the calculated oxygen concentrations were used in a reciprocal plot for the approximation of the K_M for oxygen. As shown in the inset of figure 5, the K_M for the cytochrome oxidase in the digitonin preparation was 4.5×10^{-6} M. An analogous determination on suspended particulates without digitonin revealed a K_M of 5×10^{-6} M.

Succinic-cytochrome c Reductase: As indicated in the upper curve in figure 6, succinic-cytochrome c reductase was demonstrated when succinate was added to the reaction mixture containing the particulate material. The activity of this enzyme was not observed, however, unless approximately 1 mg of glutathione was included per ml of the medium used for the isolation of the particles (2). Even though the particles were washed twice with the sucrose-phosphate-EDTA medium after isolation to remove the glutathione apparently enough glutathione remained with the particulates to cause an appreciable endogenous reduction of cytochrome c (lower curves of figs 6 and 7).

As shown by the middle curve of figure 6, SN 5949 (2-hydroxy-3-(2-methyloctyl)-1, 4-naphthoquinone) at a concentration of 3 μg per ml almost completely inhibited succinic-cytochrome c reductase activity. In figure 7 it is illustrated that almost complete inhibition of activity resulted from the addition to the reaction mixture of 1 μg of the antibiotic Antimycin A.

Succinic-cytochrome c reductase activity was consistently low in all preparations. The activity of the best preparations was about 3 % that of the DPNH-cytochrome c reductase. In view of the lability of the enzyme, no attempt was made to evaluate the total cytochrome reductive capacity of roots.

DPNH-cytochrome c Reductase: DPNH-cytochrome c reductase activity was demonstrated in both the suspended particulate material (upper curve of fig 7) and in the digitonin extracts of particles. Experiments were conducted to determine the concentrations of both DPNH and cytochrome c required for the saturation of the particulate enzyme. From the data obtained, it was calculated that the K_M for cytochrome c was 1.4×10^{-5} M and that for DPNH was 2×10^{-5} M. These constants may be compared with values of 8×10^{-6} M for cytochrome c and 1×10^{-5} for DPNH reported for a purified cytochrome c reductase from soybean leaves (5). It was demonstrated that TPNH also would function as a cytochrome reductant for the particulate enzyme, however, no complete studies were conducted with this electron donor.

Since it has been reported (21) that salts stimulated cytochrome oxidase activity in particulates of several plant species, it was desirable to determine the influence of salts on particulate cytochrome reductase. For this experiment particles were prepared as described under "Materials and Methods" and then washed once with 0.5 M sucrose and suspended in 10^{-3} M Tris(hydroxymethyl)aminomethane (TRIS) buffer at pH 7.0. The reaction mixture contained 10^{-3} M TRIS buffer at pH 7.0, 5.2×10^{-5} M cytochrome c, 1×10^{-4} M DPNH, 10^{-3} M KCN with and without KCl, NaCl, or K_2HPO_4-KH_2PO_4 at pH 7.0 at concentrations ranging from 0 to 0.1 M. These salts at the concentrations tested failed to stimulate activity. $CaCl_2$ or $MgCl_2$ at a concentration of 10^{-2} M inhibited activity approximately 50 %.

DPNH cytochrome c reductase activity was inhibited about 50 % by the addition of 0.1 ml of ethanol to the standard assay and the addition of 10 μg of Antimycin A dissolved in the ethanol exhibited no further inhibition. The addition of 3 μg of SN 5949 caused no inhibition of activity. These results may be contrasted with those obtained with succinic-cytochrome reductase where the same concentration of SN 5949 caused almost complete inhibition.

As indicated by the curve in figure 7, the capacity of tobacco root particulates to catalyze the reduction of cytochrome c with DPNH as a substrate was many fold greater than the capacity to reduce cytochrome c with succinate. It was of interest to determine whether or not the DPNH-cytochrome c reductase capacity of tobacco roots was sufficient to account for their oxygen uptake. For this experiment, the same preparations used to determine the total cytochrome oxidase capacity were utilized, and the results are presented in table II. The cytochrome c reductase capacity of the crude extract equals the cytochrome oxidase capacity and both enzyme activities are sufficient to account for the oxygen uptake. Solubilizing the enzyme complex with digitonin resulted in decreased activity of the cytochrome reductase. When 0.1 micromole menadione (2-methyl-1,4-naphthoquinone) dissolved in 25 % ethanol was included in the reaction mixture the cytochrome reductase capacity was increased approximately 50-fold and greatly exceeded the most active cytochrome oxidase preparations. The increase in activity is due to a soluble enzyme which will reduce cytochrome c in the presence of menadione and other compounds. More detailed studies have been conducted with this enzyme and will be reported in a subsequent publication.

DPNH Oxidase: A considerable number of experiments were conducted to determine whether or not particles from roots would catalyze the oxidation of DPNH. When particles were prepared and washed three times with the sucrose-phosphate EDTA me-

dium no consistent oxidation of DPNH was observed unless an electron acceptor was added to the reaction mixture. A weak oxidative rate is indicated in the middle curve of figure 8 where DPNH is added to a reaction mixture containing particles that had been washed once with the sucrose-phosphate-EDTA medium. As shown in the bottom curve of figure 8, this weak activity was inhibited by 10^{-3} M KCN. As shown by the upper curve of figure 8, a marked stimulation in activity was obtained by the addition of cytochrome c at a concentration of 5.2×10^{-6} M to a reaction mixture containing once-washed particles. When the particles were allowed to age for about 30 minutes at 0° C all the DPNH oxidative capacity was lost, but activity could be restored by the addition of a concentrated ethanol extract of tobacco roots or by the addition of either chlorogenic acid or cytochrome c. This problem receives further attention in another paper (24).

Discussion

Evidence provided by direct spectroscopic observations, spectrophotometric absorption and difference spectra, and by cytochrome c oxidase activity measurements indicate that a large portion of the classical cytochrome system is present in tobacco roots. The spectra of the cytochromes are not sufficiently definitive to determine with certainty that cytochrome c is present. The fact that digitonin extracts of roots from either vigorously growing plants or senescent plants contained 7.5-fold and 4-fold respectively greater cytochrome c oxidase capacities than would be required to account for their respiratory oxygen consumption is consistent with the view that the cytochrome system is important in the respiration process of tobacco roots. This view also is supported by the demonstration that the cytochrome reductase capacity of extracts from tobacco roots was sufficient to account for the oxygen uptake of intact roots.

The K_M value calculated for the cytochrome oxidase oxygen complex is 4.5×10^{-6} M when a digitonin extract was utilized as a source of enzyme and 5×10^{-6} M when a particulate extract was used. Yocum and Hackett (32) have tabulated the comparable K_M values determined with cytochrome oxidase preparations from various species. K_M values of 1×10^{-6} M for yeast, 3×10^{-6} M for potato slices, and 3×10^{-6} M for aroid spadix are fairly close to the value obtained with tobacco roots. The values of 3.2×10^{-8} M for cytochrome oxidase in Aerobacter and 2.4×10^{-8} M for a purified cytochrome oxidase from animal tissue reported by Longmuir (17) are far lower than the values obtained for the enzyme from tobacco. The K_M of the phenol oxidase-oxygen complex using extracts from tobacco was determined in a manner somewhat analogous to that used in arriving at the K_M for the cytochrome oxidase-oxygen complex. The details of this determination will be reported in a later publication (24). A value of 1.5×10^{-5} M for phenol oxidase was obtained which is about 3-fold greater than that obtained for cytochrome oxidase. From these data it is apparent that the concentration of oxygen required for phenol oxidase to function at one half the maximum rate is three times that required for cytochrome oxidase to function at one half the maximum velocity. This consideration together with the fact that a relatively large activity of cytochrome oxidase was demonstrated in tobacco roots suggests that cytochrome oxidase may account for a large portion of the terminal electron transport at least under conditions where oxygen concentrations are low. Fritz and Beevers (6) concluded that the activity of cytochrome oxidase was adequate to account for the oxygen uptake in pea shoots and roots, and in wheat or barley shoots, but was insufficient to account for the oxygen consumption by wheat or barley roots. This latter conclusion should be reexamined in view of our finding and also those recently reported by Simon (23) that dispersion with digitonin greatly increased the cytochrome oxidase capacity of particulate extracts.

Webster (31) reports a 43 % inhibition of the respiration of tobacco roots by 95 % carbon monoxide in the dark and a 7 % inhibition of them in the light. The difference is generally considered to be due to the inhibition of cytochrome c oxidase by carbon monoxide in the dark and a reversal of the inhibition by light. In the experiments conducted in our laboratory with tobacco root initials, the respiratory rate was not inhibited by 95 % carbon monoxide. In parallel experiments, however, the respiration of soybean roots was inhibited by 95 % carbon monoxide in the dark and the inhibition was reversible by light. The reason CO did not inhibit the respiration of the tobacco root segments has not been determined.

It is apparent from the data in table I that digitonin is far superior to sodium cholate for the extraction of cytochrome oxidase activity from particles. The increased cytochrome oxidase activity obtained by extraction of particulates with digitonin may indicate that dispersion of particulates is necessary in order to expose all the available enzyme to exogenous substrates as was suggested by Simon (23).

The presence of a succinic-cytochrome reductase, inhibited by low concentrations of SN 5949 and Antimycin A, indicates that tobacco metabolizes succinate through a system similar to those reported for other higher plant species (8, 15). These compounds are reported to manifest their inhibitory action through the Slater factor (25, 8, 15). Chance and Williams (3) report spectroscopic evidence that this factor functions between cytochromes b and c in animal mitochondria.

A DPNH-cytochrome c reductase is present in tobacco roots in amounts adequate to account for the respiratory rate (table II). Hackett (8) found that Antimycin A did not inhibit DPNH-cytochrome reductase in potato, while Humphreys and Conn (15) found that both Antimycin A and SN 5949 inhibited the enzyme in lupine mitochondria. The failure of Antimycin A and SN 5949 to inhibit DPNH-cytochrome c reductase in tobacco indicates that the

Slater factor is not in the electron transport pathway between DPNH and cytochrome c.

Summary

A study has been conducted to determine the properties and the relative significance of the cytochrome system in the roots of tobacco plants. By use of a direct spectroscopic method with particulates and spectrophotometric studies with digitonin extracts of root particulates, absorption bands have been demonstrated that are characteristic of cytochrome oxidase and cytochromes b or c or perhaps both. An evaluation of the cytochrome c oxidative capacity of extracts has provided evidence that roots contain sufficient activity of this enzyme to account for the respiratory oxygen uptake by intact roots.

The Michaelis constant for the cytochrome oxidase-oxygen-complex using digitonin extracts of roots has been estimated to be 4.5×10^{-6} M. The comparable K_M for particulates without digitonin was estimated to be 5×10^{-6} M. These values are approximately one third that estimated for the phenol-oxidase-oxygen complex in tobacco root extracts.

Succinic-cytochrome c reductase activity which was inhibited by Antimycin A and SN 5949 (2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone) was demonstrated in particulates from tobacco roots. Also the activity of a reduced pyridine nucleotide cytochrome c reductase was demonstrated in particles and it was shown that the cytochrome c reductase capacity of this enzyme was sufficient to account for the respiratory oxygen uptake of roots. Tobacco root extracts failed to oxidize DPNH at an appreciable rate unless some electron acceptor such as cytochrome c menadione or chlorogenic acid was added to reaction mixtures.

Literature Cited

1. Bhagvat, K. and Hill, R. Cytochrome oxidase in higher plants. New Phytologist 50: 112–120. 1951.
2. Bonner, W. D. Succinic dehydrogenase. In: Methods in Enzymology. I, S. P. Colowick, and N. O. Kaplan, eds. Pp. 722–729. Academic Press, New York 1955.
3. Chance, B. and Wiliams, G. R. Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. Jour. Biol. Chem. 217: 429–438. 1955.
4. Cooperstein, S. J. and Lazarow, A. A microspectrophotometric method for determination of cytochrome oxidase. Jour. Biol. Chem. 189: 665–670. 1951.
5. Evans, H. J. Studies on cytochrome reductase in higher plants. Plant Physiol. 30: 437–444. 1955.
6. Fritz, G. and Beevers, H. Cytochrome oxidase content and respiratory rates of etiolated wheat and barley seedlings. Plant Physiol. 30: 309–317. 1957.
7. Goddard, D. R. Cytochrome c and cytochrome oxidase from wheat germ. Amer. Jour. Bot. 31: 270–276. 1944.
8. Hackett, D. P. Pathways of oxidation in cell free potato fractions. Plant Physiol. 31: 111–118. 1956.
9. Hackett, D. P. Pathways of oxidation in cell-free potato fractions. II. Properties of the soluble pyridine nucleotide oxidase system. Plant Physiol. 33: 8–13. 1958.
10. Hartree, E. F. Haematin compounds. In: Modern Methods of Plant Aanalysis. IV. Pp. 197–245. Springer-Verlag, Berlin 1955.
11. Hartree, E. F. Cytochromes in higher plants. In: Advances in Enzymology and Related Subjects in Biochemistry. F. F. Nord, ed. Vol. 18. Pp. 1–64. Interscience Publ., New York 1957.
12. Hill, R. and Scarisbrick, R. The haematin compounds of leaves. New Phytologist 50: 98–111. 1951.
13. Horecker, B. L. and Heppel, L. A. The reduction of cytochrome c by xanthine oxidase. Jour. Biol. Chem. 178: 683–690. 1948.
14. Horecker, B. L. and Kornberg, A. The extinction coefficients of the reduced bands of pyridine nucleotides. Jour. Biol. Chem. 175: 385–390. 1948.
15. Humphreys, T. E. and Conn, E. C. The oxidation of reduced diphosphopyridine nucleotide by lupine mitochondria. Arch. Biochem. Biophys. 60: 226–243. 1956.
16. Keilin, D. On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. Proc. Royal Soc. (London), Series B 98: 312–329. 1925.
17. Longmuir, I. S. Respiratory rate of bacteria as a function of oxygen concentration. Biochem. Jour. 57: 81–87. 1954.
18. Lowry, O. H., Rosenrbough, Nira J., Farr, A. L. and Randall, Rose J. Protein measurements with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275. 1951.
19. Lundegardh, H. On partial oxidation of the cytochrome system in the presence of cyanide. Physiol. Plantarum 8: 95–105. 1955.
20. Martin, E. M. and Morton, R. K. Haem pigments of cytoplasmic particles from non-photosynthetic plant tissues. Biochem. Jour. 65: 404–413. 1957.
21. Miller, G. W. and Evans, H. J. The influence of salts on the activity of particulate cytochrome oxidase from roots of higher plants. Plant Physiol. 31: 357–364. 1956.
22. Miller, G. W., Evans, H. J. and Sisler, E. The properties of cytochrome oxidase in cholate extracts from soybean roots. Plant Physiol. 33: 124–131. 1958.
23. Simon, E. W. The effect of digitonin on the cytochrome c oxidase activity of plant mitochondria. Biochem. Jour. 69: 67–74. 1958.
24. Sisler, E. C. and Evans, H. J. Electron transport mechanisms in tobacco roots. II. Reduced pyridine nucleotide oxidative pathways. Tobacco Science (In press).
25. Slater, E. C. The components of the dihydrocozymase oxidase system. Biochem. Jour. 46: 484–499. 1950.
26. Smith, Lucile. Reactions of cytochrome a and a_3. I. Studies of oxidation and reduction of the pigments in a purified preparation. Jour. Biol. Chem. 215: 833–846. 1955.

27. SMITH, LUCILE and CONRAD, HELEN. A study of the kinetics of the oxidation of cytochrome c by cytochrome c oxidase. Arch. Biochem. Biophys. 63: 403–413. 1956.
28. UMBREIT, W. W., BURRIS, R. H. and STAUFFER, J. F. Manometric Techniques. Pp. 70–72. Burgess, Minneapolis 1957.
29. WAINIO, W. W. and ARONOFF, M. Effect of some surface active agents on cytochrome oxidase. Arch. Biochem. Biophys. 57: 115–123. 1955.
30. WEBSTER, G. C. The occurrence of a cytochrome oxidase in the tissues of higher plants. Amer. Jour. Bot. 39: 739–745. 1952.
31. WEBSTER, G. C. The effect of carbon monoxide on respiration in higher plants. Plant Physiol. 29: 399–400. 1954.
32. YOCUM, C. S. and HACKETT, D. P. Participation of cytochromes in the respiration of the aroid spadix. Plant Physiol. 32: 186–191. 1957.

NEWS AND NOTES

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Executive Committee Meeting, University of Indiana, Bloomington, Indiana, August 24, 1958*: The Executive Committee Meeting was opened at 8:10 P.M. by President Goddard. Present were: H. Beevers, J. Bonner, W. D. Bonner, Jr., H. A. Borthwick, A. H. Brown, F. P. Cullinan, A. W. Galston, D. R. Goddard, S. B. Hendricks, N. Higinbotham, R. W. Krauss, W. E. Loomis, B. S. Meyer, G. R. Noggle and K. V. Thimann.

The minutes, as published in the January issue of PLANT PHYSIOLOGY, were approved.

The Executive Secretary-Treasurer, Dr. G. R. Noggle, presented his annual report on the status of membership and finances. Dr. Noggle reported that membership in the Society continues to increase, especially in student memberships. The increase in membership has been accompanied by an increased number of subscriptions to the Journal, but owing to a drop in foreign subscriptions, the total mailing of the Journal has dropped from last year. (2,873 copies in 1957, 2,832 copies in 1958.) Dr. Noggle reported that the Society continues to be in a conservative and favorable financial position and will be able to maintain services to its members during the year ahead. These thoughts are reflected in the net total worth of the Society which increased from $79.98 to $81,227.12 during the past year.

Dr. Noggle's Report which was unanimously approved is detailed below:

MEMBERSHIP

	July 1, 1957	July 1, 1958
Life Members	34	33
Corresponding Members	19	19
Canadian Members	38	46
U. S. Members—Regular	1068	1081
Student	78	128
Foreign Members	122	145
	1359	1452
Less those included in other groups	5	5
Total	1354	1447

SUBSCRIPTIONS

U. S. Subscriptions	Vol. 32. 426	Vol. 33. 435
Foreign Subscriptions	Vol. 32. 1093	Vol. 33. 960
Total	1519	1395

SUMMARY OF CASH ACCOUNTS

Checking Account, July 1, 1957	$15,200.50
Savings Account, July 1, 1957	27,679.48
Cash Balance, July 1, 1957	$42,879.98
Receipts 1957-58	33,320.75
Total	76,200.73
Expenditures 1957-58	37,273.61
Cash Balance, July 1, 1958	$38,927.12
Cash on hand July 1, 1958	
Checking Account	$15,820.41
Reserve Account	23,106.71
Total	$38,927.12

SUMMARY OF ENDOWMENT ACCOUNT
(*Life Membership and General Endowment Fund*)

Balance, July 1, 1957	$6345.72
Income, 1957-58	
Sales of back numbers of Plant Physiology	1286.50
Investments	1028.88
From Barnes (Loomis)	150.00
Total	$8811.10
Disbursement (Purchase of Pot. P. and EL.)	5456.15
Balance, June 30, 1958	$3354.95

The Editor of PLANT PHYSIOLOGY, Dr. A. H. Brown submitted a report on his first year as editor and proposed a budget for 1958–59. The substance of this report, which was approved unanimously, is here reproduced.

The transition of editorship from one office to another was greatly facilitated by the excellent cooperation of Prof. Goddard and his assistant, Mrs.

Valentine. For this the present editor is very grateful.

During the past year the editor set his sights on four principal objectives: 1) maintaining the standards of our journal at least at the level which had been achieved by his predecessor, 2) speeding up the processing of manuscripts so as to shorten the time between submission of a paper and its appearance in print, 3) improving insofar as possible the literary quality of contributions which we publish, and 4) learning the routine and not so routine aspects of editorship well enough to carry out the various duties with reasonable efficiency and still have time and energy remaining for other activities.

As far as is apparent at this time, the general character of our journal has undergone no very great change in the past year. Admitting the likelihood that the present editor may not be viewing the journal from the best possible vantage point, it seems that the quality of the contributions now being published is largely comparable with what appeared in previous volumes under Prof. Goddard's term of office. The editor is continually impressed by the fact that the standards of our journal depend not so much on his own efforts but chiefly on the quality of manuscripts submitted —secondarily on the recommendations of reviewers who aid the editor in the selection of manuscripts for publication and especially on the reviewers' suggestions and comments aimed at helping authors improve their papers before final processing. The editor appreciates—no doubt better than can anyone else—the time and thought which over 150 reviewers have devoted to this task of manuscript evaluation during the past year.

The second objective of speeding up publication has been disappointingly elusive. Modified arrangements within the Press promise to help in the future. But it has been a source of some discouragement that more could not be done within the editorial office to promote more rapid publication. The current situation is shown in the following tabulation:

Time Study on Published Manuscripts

Average time from receipt to publication	6.2 mo. (25 wks)
from receipt through review	6.3 wks (44 days)
from review to decision	1.1 wk (7.8 days)
from decision to return by author	3.9 wks (27.5 days)
from return to publication	3.2 mo. (13 wks)

The editor now recognizes that his earlier hope to improve the literary quality of the journal was somewhat naive. He has tried to impress upon contributors his conviction that scientific writing need not be so very different from other kinds of prose except for the employment of a technical vocabulary. He has heartily endorsed reviewers' suggestions for improving the clarity and precision of expression in manuscripts in process. He has even indulged in limited rewriting of a manuscript or two, but he is not in a position to do much more than this.

On the fourth objective, that of running the journal with reasonable immunity to ulcers and with time to spare, it perhaps is too early to appraise the extent of our progress. However, there does seem to be cause for optimism. The editor was fortunate beyond all expectation in having as his assistant Dr. Mary A. Swaebly-Ehrlich, who has done an admirable job in running the mechanical aspects of the editorial office both efficiently and dependably. Her skillful assistance is a major cause for the favorable prognosis which now seems warranted.

Policy matters of an editorial nature, format and style changes, and certain procedural topics are properly the concern of the editor and the Editorial Board. Prior to the annual meeting of the Editorial Board the editor has no major policy changes to announce. One alteration in the journal which may have been noted is the disappearance of a "brief paper" section. Short contributions now receive the same treatment as longer manuscripts—evaluation by two reviewers and, upon acceptance, publication in turn according to date of receipt.

Traditionally an editor's report ought to contain a tabulation of the fate of submitted manuscripts. The following statistics should meet this requirement. This summary, up to date on 15 August, 1958, includes only papers handled throughout by the present editorial office, except for item E which includes some papers processed by the previous editor.

A. Manuscripts received, 13 August, 1957 to 13 Aug. 1958		154
B. Accepted for publication		77
with little or no revision	59	
after revision	18	
C. Returned for major revision		34
D. Rejected or withdrawn		28
rejected	19	
withdrawn	9	
E. Published (Jan. through July)		65
F. Papers now in process		89
awaiting reviewers	8	
in reviewers' hands	16	
awaiting decision	9	
awaiting return by author	32	
at the press	24	
(Sept.)	18	
(Nov.)	6	

A comparison of our present operations with those of previous years gives rise to the following statistics:

	1954	1955	1956	1957	1958
Papers received	157	150	151	254	154
Papers accepted			83	172	77
Papers rejected or withdrawn	41 %	21 %	18 %	13 %	18 %

Apparently the spectacular increase in number of submitted manuscripts during 1957 was not indicative of a continuing trend. Its cause is unexplained.

The projected expenses of the editorial office were estimated a year ago by this committee at $4100.00. (Printing and mailing costs are eliminated from consideration because these are handled directly in the office of the executive secretary-treasurer.) The editor nearly succeeded in spending this amount. The following tabulation includes a proposed budget for the editor's office for the next 12 months.

Budget year	1957–58		1958–59
	approved	actual	proposed
Editorial assistant	$2500	$2480.00	$2901.60
Editor's stipend	1000	1000.00	1000.00
Supplies	300		
Postage		378.00	300.00
Supplies		81.91	75.00
Miscellaneous		11.25	20.00
Contingency	300		
	$4100.00	$3951.16	$4296.60

A budget of $4300 is requested for 1958–59. The increase is justified by the fact that the position of editorial assistant was figured on a one-half time basis but turned out to be three-fourths time. The present editorial assistant has been paid at this rate since 1 Jan. of this year. The editor recommends continuation of this three-fourths time position as indicated.

The editor makes no predictions or promises concerning the immediate future of our journal beyond the simple reiteration of his intention to do the job as well as he can and to be grateful for all criticisms and constructive suggestions for improving our journal.

There was some discussion of the proposed budget. Dr. Goddard suggested that the budget for PLANT PHYSIOLOGY should combine the editor's and printer's budgets. This suggestion was acted on favorably and a budget of $25,000 was approved by the Executive Committee. This budget does not include the cost of printing supplements to the Journal.

The Secretary reported on the nomination and election of officers. Two hundred and seventy four nomination ballots were returned resulting in 374 names to be considered for the election ballot. Those names having the highest number of nominations were placed on the election ballot, of which 640 were returned. The elections resulted in the following officers: President, S. B. Hendricks; Vice-president, A. C. Leopold; Member Exec. Committee, H. A. Borthwick; Members of the Editorial Board, P. J. Kramer and A. S. Crafts.

The Secretary reported on correspondence with the Secretary-General of the 9th International Botanical Congress and with members of the Executive Committee relating to the Society's plans regarding meeting with AIBS in 1959. In spite of a mail ballot which had indicated the majority of the executive committee being opposed to meeting with the AIBS in 1959, the year of the International Congress, a minority opinion in favor of meeting with the AIBS was vigorously expressed. Because of the irreconcilable difference of views within the Executive Committee, it was decided to take this question to the membership.

Dr. Victor A. Greulach, chairman of the committee on Professional Status and Training, submitted a written summary of the recent activities of the committee which culminated in the committee's report, "Plant Physiologists in Industry," being published as a supplement to volume 32. The Executive Committee expressed great satisfaction with the report and are grateful to Dr. Greulach and the members of his committee for their considerable efforts. With the publishing of this report, the Committee on Professional Status and Training is dissolved.

Dr. B. S. Meyer, representative on the AIBS Governing Board reported that the activities of the AIBS were increasing to such a point that the existing income from member societies was no longer adequate. The income which supports the AIBS has been derived from dues contributed by member societies, and collected at the rate of $1.00 per member for the first 1000 members only. The AIBS Governing Board has indicated that it hopes member societies will voluntarily remove the 1000 member limit (as has been done in one or two cases already). The Executive Committee passed a motion that the American Society of Plant Physiologists contribute dues to the AIBS at the rate of $1.00 per member.

Dr. M. W. Parker, representative on the council of the AAAS reported that the activities of the council were published regularly in Science and that he had nothing further to report.

Dr. R. W. Howell, Representative on the Agricultural Research Institute, reported that he had not participated in any of the activities of the Institute as yet. Dr. Howell expected to attend a meeting of the Institute in the late fall of 1958 and would report on this meeting in due course.

Dr. H. A. Borthwick reported that he had transacted no business in his capacity as Representative on the Editorial Board, American Journal of Botany.

Dr. J. van Overbeek, representative on the American Standards Association Sectional Committee on common names for pest control chemicals (Section K62), reported that the work of the committee is light and consists primarily of passing on new words coined for chemicals commonly used in pest control. Names, usually proposed by the company producing the chemical, are adopted by the committee when they make sense and do not violate any scientific principles. The committee performs a very useful service in rejecting names that are unreasonable. Dr. van Overbeek cited an example where the committee had recently served such a useful "watchdog" function.

The representative on the Joint Committee on Grassland Farming, Dr. Milton A. Sprague, submitted a report of a recent Executive meeting of the Joint Committee. In this meeting the name of the Committee was changed to "American Grassland Council." Dr. Sprague reported that the membership of the American Grassland Council has been increas-

ing and that the Council hopes that their close and cordial relationship with our Society will continue and be cemented more firmly.

A report from Dr. D. B. Anderson, representative to the National Register of Scientific Personnel, stated that operations have been proceeding satisfactorily from the standpoint of the register, but from the standpoint of our society there is some concern at the long delay which occurs between the sending out of the questionnaires and their return. Dr. Anderson reported that the register was under-staffed but there was hope for improvement in this regard.

Dr. F. W. Went reported that the committee forming an International Society of Plant Physiologists has, through correspondence with the different representatives of the national Societies, come to agreement on a tentative constitution and officers. The officers are: Dr. T. A. Bennett-Clark, President; Dr. H. Burstrom, Vice-president; and Dr. B. Kok, Secretary.

The Executive Committee, on the recommendation of the Editor of PLANT PHYSIOLOGY, elected Dr. D. R. Goddard and Dr. S. Gordon to the Editorial Board (5-year appointment) and President Goddard reappointed Dr. F. P. Cullinan to the Board of Trustees (3-year term).

The value of the pamphlet, "Plant Physiology as a Career" came up for discussion. In view of the continued interest in the pamphlet from high schools and colleges throughout the U.S., and because the pamphlet is now outdated, it was decided to rewrite the pamphlet. President Goddard will appoint a committee to do so.

Dealing with new business the Executive Committee discussed at length the revision of the ASPP's investment policy. The members of the board of trustees have had a thorough appraisal of our investments made by highly competent financial experts. A revision of our investment policy is in order and such a revision is left in the capable hands of the Executive Secretary-Treasurer and the members of the board of trustees.

A discussion of the establishment of Emeritus standing in the Society, a change in our by-laws, resulted in the following proposition to be submitted to the members at the annual business meeting: *A member reaching age of retirement and who has been a member ten years or more may apply to be admitted to Emeritus membership. Such a member will not pay dues, will not receive the Journal, but will retain all other rights of membership.*

In order to gain more stable membership of the executive committee it was proposed to place before the membership the following: *Each local section will elect a representative to the executive committee to serve for a three-year term. The President of each section will arrange the staggering of the election of these representatives through a schedule arranged by the Secretary.*

Other business transacted by the committee included: 1) Authorization for the executive secretary-treasurer to increase his office help budget from $800 to $1000. 2) Authorization for President Goddard to consider personnel of a committee, or committees, to examine the suitability of making application to the NSF for funds to support a program which would allow college teachers to study and work in individual laboratories during the summer.

Dr. Galston announced that the executive committee of the Northeastern Section had invited members in Pennsylvania and Delaware to join this section.

President Goddard announced the recipient of the Stephen Hales award (Prof. F. W. Went) and the Charles Reid Barnes Life Membership (Dr. P. Boysen-Jensen).

The meeting was adjourned at 11:30.—Respectfully Submitted, WALTER D. BONNER, JR., *Secretary.*

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Annual Business Meeting, August 26*, 1958: The annual business meeting was opened by President Goddard at 11:30 A.M. The Executive Secretary-Treasurer, Dr. Noggle, reported the Society to be in a conservative and favorable financial position and that the membership continued to increase. The Editor of the Journal, Dr. Brown, did not comment except to announce that he had no formal report to make other than what had been said at the Executive Committee meeting. The Secretary reported briefly on the election of officers.

During a discussion concerning whether or not our Society should meet with the AIBS at Pennsylvania State University in 1959, Dr. Bandurski pointed out that the enthusiast could look forward to four weeks of continuous meetings in 1959. After a lively discussion a vote showed the majority of members wished to meet with the AIBS in 1959. Dr. A. Brown moved that the Society hold its 1959 Business meeting in Montreal in connection with the International Botanical Congress. This proposal was seconded and passed. The change in by-laws to allow the establishment of Emeritus status was voted on and passed as was the proposal to stabilize the executive committee membership by election of representative by local section. This latter proposal, however, was amended to include the statement that members of sections who are not members of the Society cannot serve as representatives on the executive committee. Following a discussion, the former ruling that student members are not entitled to vote was rescinded.

Dr. Galston proposed a vote of thanks to Dr. C. O. Miller for his efforts which resulted in the extremely satisfactory arrangements for our meeting. There being no further business, the meeting was adjourned at 12:10 P.M.—Respectfully submitted, WALTER D. BONNER, JR., *Secretary*

BIRTHDAY GREETINGS: On the 19th of this month Prof. Charles A. Shull celebrates his 80th birthday. Just 35 years ago he was our Society's first president. He also served as Editor-in-chief of this Journal and has been honored by the society with the Stephen Hales Prize and the Charles Reid Barnes Award. Some of us know him for his many contributions to our science and our Society; others know him personally as a teacher or research colleague and love him as a friend. All of us wish him well and send birthday greetings.

INFORMATION AND INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

I. Submission and Processing of Manuscripts

1. Send the original copy of the manuscript, the first carbon, and two copies of the illustrative material to the Editor, Dr. Allan H. Brown, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota. The author should retain a carbon copy of the manuscript and his original photographs or drawings to insure against loss and to consult in case of need.

2. The Manuscript Will be Sent out for Review by two competent reviewers familiar with the field of research covered by the paper. These reviewers may or may not be members of the Editorial Board. In case of marked disagreement between reviewers, the Editor will appoint a referee, usually a member of the Board, to make a recommendation for the Board. Usually two months suffices for review and decision of the Editorial Board.

3. Manuscripts Returned to the Author for Revision should be sent back to the Editor within two months or the paper will be published under the date of receipt of the revised manuscript and not the original date.

4. Galley Proof will be sent to the designated author (See below; Part II, Sec. 4) by the printer, but it should be returned directly to the Editor. Correct all errors clearly, and return the galley promptly. Except for corrections of printer's errors, authors will be billed at cost for alterations in the galley proofs involving author's changes from manuscript copy, but not for corrections of printer's errors.

5. Reprint Orders should be sent to the Editor on the form provided with the galley proof, even if formal requisitions or purchase orders for institutional agencies follow later. Late orders for reprints entail extra work and expense by the printer for which authors must be charged. Since type is destroyed immediately after printing, it is impossible to supply reprints after papers are published.

6. Abstracts for Biological Abstracts are also to be returned to the Editor with the corrected galley. Authors should submit their typed abstracts on the form forwarded with the galley. The Editor will insert proper volume and paging.

II. Preparation of the Typescript

When a manuscript is accepted by the Editor for publication, the Editor and his staff must prepare the typescript for the printer by proofmarking the submitted copy. In proofmarking, the copy is put in a form consistent with the policy of the journal, but at the same time the individual style and personal preferences of the author are retained as far as possible. The author can do much to assure himself that his published articles will be free from mechanical errors by carefully preparing his typescript, giving special attention to the form used by the journal, especially in regard to tables, figures, and literature citations.

1. Typing. The typescript must be *double spaced throughout*, including footnotes, figure legends, tables, and literature citations. Only double spaced copy can be proofmarked for the printer, thus eliminating costly corrections. Liberal side margins should also be allowed for proofmarking. *Each table, the figure legends and the* Literature Cited *section should be typed* (double spaced) *on a separate piece of paper,* since they are specially set by the printer in a type-form different from that of the body of the manuscript.

2. Length. The allowable length of papers is 10 printed pages, including figures, tables, and literature citations. Authors will be billed $20.00 per page for overrun. Approximately four double spaced typewritten pages will equal one page of printed type.

3. Format. Consult recent editions of PLANT PHYSIOLOGY for proper placement of headings, paragraph headings and subheadings, and the use of abbreviations. Sources of serious error are eliminated if the typescript conforms to the established format of published papers.

4. First Page, Footnotes, and Acknowledgments. On the first page of the typescript, above the title, the author should indicate the name and address of the person to whom editorial correspondence, including the galley proof and reprint order form, is to be sent. The name and address of the institution follows the name of the author(s) directly below the title. One or more footnotes are included at the bottom of the first page. The 1st footnote (indicated by superscript 1 after the title) gives the date of receipt of the manuscript or of the revised manuscript (see above); the 2nd footnote credits the source of financial support (if any); and additional footnotes give the address of the author(s), if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used. If footnotes must be used in the body of the text, they should be numbered consecutively with Arabic numerals and typed on the bottom of the page in which reference to the footnote is made. Acknowledgments are not included as a footnote, but they are given at the end of the body of the manuscript following the Summary and preceding the Literature Cited.

5. Scientific Names. The complete scientific name (including genus, species and the authority for the binomial) of all experimental plants should be included in every manuscript. Authors are referred to the most recent International Code of Botanical Nomenclature for the rules governing the use of scientific names.

6. UNDERLINING. Leave all headings unscored and scientific names without underlining. The Editor will proofmark these items in accordance with the policy of the journal. In general, do not underscore any lines.

7. NUMBERS. Numerals, cardinal and ordinal, are written out when they begin a sentence. In the body of the text cardinal numbers and the contracted form for ordinals are always used, i.e., 1st, 10th, 25th, 1, 5, 13, 25. For values of less than one, a zero should be inserted before the decimal point, i.e., 0.7 g, not .7 g. Fractions are normally written out, and are not hyphenated; for example, one fourth, two thirds, two and one half, except in tables and graphs where 1/4, 2/3, 2 1/2 are appropriate. It is best, however, to avoid fractions whenever possible and substitute the decimal form, i.e., 0.5, 0.1 and 2.5.

8. TABLES. Authors should study carefully the form of tables used in recent issues of PLANT PHYSIOLOGY and set up their tables in a form consistent with the established policy. Tables should be preplanned to fit 1 or 2 printed columns (3 inches and 6⅛ inches wide respectively) and not to exceed 8½ inches in height, including heading, body and footnotes. Tables may not be rotated 90°. On the average, about 52 pica typewriter spaces fill 1 line of a 3 inch column of type of the size normally used in tables, and 10 lines require 1 inch of the available vertical space. Tables are numbered consecutively in Roman numerals, and footnotes are referred to by the use of the asterisk, double asterisk, dagger, double dagger, and section, in this sequence, as needed. *All explanatory material should be placed in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity. When typing tables, *double space throughout,* including headings and footnotes to facilitate marking the copy for the printer. Tabular data as well as numerical values reported in the text should be checked for the proper number of significant figures. Frequently authors report more digets than are significant.

9. FIGURES. Figures are of two kinds, half-tones and line drawings (including graphs). A very good photograph printed on glossy paper and in sharp focus throughout is necessary for satisfactory reproduction. Limit the photograph to desired visual details. Trim off irrelevant portions and minimize blank space. The author(s) name, the figure number, and the top of the figure should be indicated on the reverse side of all illustrative material. *Do not send original drawings,* but submit only properly mounted and grouped black and white photographs for reproduction. For purposes of manuscript review, it is satisfactory to furnish verifax, blue print or other reproduction form of figures. The prints from which the printers' cut will be made then need not be sent through the mails repeatedly.

All illustrations should be mounted with rubber cement on white cardboard (if a number of pictures are grouped together) or very stiff white paper (suitable for individual pictures) and they should not exceed a total measurement of 9 × 11 inches to eliminate the necessity of special packaging and handling. Mounting even individual illustrations precludes loss and possible damage from paper-clipping the picture directly to the bulk of the manuscript.

As far as possible all figures should be grouped and mounted close together on white cardboard. Halftone and line drawings must be placed in separate groups since they are reproduced by different processes. Grouping will result in a considerable saving in publication cost to the journal. When several figures are grouped together, provision should be made on the illustration or in the legend for easy identification by the reader.

The author should plan in advance for a legible finished copy when he is preparing his illustrations. Figures are reproduced the same size as the submitted photograph or they are reduced. The Editor will proofmark the illustrations to determine the actual size of the printed figures, but the author should plan for *maximum reduction,* keeping in mind that the smallest number or letter on the *reduced* illustration should be no smaller than 1 mm to assure legibility. All illustrations must fit either a 3 inch or 6⅛ inch column and not exceed 8½ inches in height. If possible, allowance should be made so that the figure legend can be accommodated at the bottom of the printed page. The legend, however, may not be typed on the illustration, but must be typed (double spaced) on a separate piece of paper, since it is set in type by the printer and is not reproduced by the engraver.

Line drawings and graphs should be intensely black on white, and the submitted photographs should be in sharp focus and without blemish. Generally the appearance of a graph is improved if the top and right side is boxed in by lines of the same weight as the abscissa and ordinate. Guided lettering is preferable to hand lettering or typing on figures and graphs. In graphs, special care should be taken to allow for maximum reduction. Symbols, such as triangles, circles and squares can become indistinct upon reproduction if they are not sufficiently large in the original. However, overly large symbols, thick lines or very heavy numerals may also be unsightly or indistinct after reduction.

10. LITERATURE CITED. This section of the typescript requires a great deal of proofmarking for the printer even though it may be correct in every detail. Therefore it is appreciated if authors exercise special care in the preparation of their bibliography. Since the literature citations are set in a typeform different from that used for the body of the manuscript, they should be typed (*double spaced*) on a separate piece of paper. Consult recent issues of PLANT PHYSIOLOGY for the proper form and for abbreviations for journals. Note the limited use of capitals as well as the correct form for citing books and chapters in books. Check to make certain all citations are complete, including title, volume numbers, inclusive pages, and the year of publication. Foreign titles should be rechecked for diaeresis and other markings. The citations should be numbered and listed in alphabetical order (*not* in order of reference) and indi-

cated in the body of the text by Arabic numerals enclosed in parenthesis. Recheck to make certain that all citations are referred to in the text. Unpublished data and personal communications are *not* acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press or in preparation may be so designated in the LITERATURE CITED. An article is not properly referred to as "in press" unless it has been *accepted* for publication. The journal in which an in-press article will appear should be included in the literature citation.

11. ERRATA. If necessary, an errata will be included in the final issue of each volume. Authors are therefore requested to call the attention of the Editor to any significant errors in their published manuscripts.